THE EFFECTS OF PSYCHOLOGICAL STRESS ON AN ANIMAL MODEL OF MULTIPLE SCLEROSIS, THEILER'S VIRUS INDUCED DEMYELINATION

A Dissertation

by

AMY NICOLE SIEVE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Psychology

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ABSTRACT

The Effects of Psychological Stress on an Animal Model of Multiple Sclerosis, Theiler's Virus Induced Demyelination. (December 2004)

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Multiple Sclerosis (MS) is the most common demyelinating condition of the central nervous system (CNS), resulting in paralysis and death. The etiology of MS is unknown. However, genetics, exposure to a pathogen, psychological stress and gender are all implicated in the onset and progression of the disease. An animal model of MS, Theiler's virus (TMEV) infection, causes a biphasic disease. An early CNS viral infection, if allowed to persist within the CNS, is followed by a chronic CNS autoimmune demyelinating condition that is similar to MS. The development of Theiler's Virus Induced Demyelination (TVID) is under genetic control: SJL mice are highly susceptible to viral persistence and TVID while CBA mice have an intermediate susceptibility. Chronic restraint stress (RST) administered during the first four weeks of TMEV infection influenced the subsequent development of TVID differentially across strain and sex of mice. TVID was exacerbated by RST in male and female SJL mice, but in the CBA strain, TVID was alleviated by RST in male mice only. This pattern of results in SJL and CBA mice could be seen in the chronic phase of TVID on

multiple dependent measures: body weights, behavioral signs of the chronic phase, rotarod performance (an automated measure of motor abilities), and inflammation, demyelination, and axonal loss within the spinal cord. The exacerbation of TVID in SJL mice provides some of the first experimental evidence that coincides with reports of stress precipitating the onset of MS in human patients. The sex dependent alleviation of TVID in CBA mice illustrates the complex interaction between genetic predisposition, gender, stress, and exposure to a pathogen that has been proposed for the development of MS.

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CHAPTER I

AN INTRODUCTION TO THE PROBLEM

Etiology of Autoimmunity

Psychological stress is something that pervades our everyday lives. The stressors that we are exposed to and the way in which we respond to those stressors may in part determine the functioning of our immune system and our susceptibility to disease. Along with infectious disease processes, the development of autoimmune disease has been linked to the activity of our bodies' physiological systems that respond to psychological stress. Individual differences in the basal functioning of these stress systems, as well as responses to specific stressors, have been shown to have a profound impact on the susceptibility to and progression of autoimmune diseases in animals and humans. In the human autoimmunity literature, periods of life stress are reported to precipitate the onset, relapse, and progression of many autoimmune diseases. Animal models of autoimmune diseases have likewise unveiled a significant effect of psychological stressors on the development of models of such diseases.

Autoimmune diseases affect 5-7% of North Americans and Europeans, decreasing the quality of life and frequently causing death in those that suffer from them (Schedlowski & Tewes, 1999; Martino & Adorini, 1999). Autoimmune

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This dissertation follows the style of the Journal of Neuroimmunology.

diseases vary widely in their symptomatology. The commonality that exists among them is the immune system acting in an aberrant fashion, attacking the individual's own body, causing pain, blindness, paralysis, or organ failure. A topic that is of interest to researchers is what triggers the immune system to act in this fashion - to attack *self*-antigens in addition to foreign antigens. Although the exact etiology of most of these diseases remains unclear, genetic and environmental factors have both been implicated. The predominance of autoimmune disorders in women has been linked to both sex hormones and differences in the functioning of stress systems. Genes coding for various aspects of the immune system, exposure to pathogens, and lifestyle factors have all been linked to the onset and progression of these diseases. Psychological stress is one environmental factor that frequently occurs prior to the onset and relapse of autoimmune diseases and may play an important role in the development of autoimmunity. In order to investigate what makes the immune system engage in the development of an autoimmune disease, the normal functioning of the immune system must be understood.

Overview of the Immune System

The immune system has been theorized to have developed to maintain homeostasis. For the immune system, threatened homeostasis occurs when foreign pathogens enter the body. It is designed to locate and eliminate foreign bacteria and parasites as well as dead or damaged self tissue, which may exist due to necrosis, apoptosis, viral infection, or cancerous activity. The immune

system's activity is held in check by molecules secreted by its own cells (e.g. cytokines and chemokines), as well as hormones secreted by other organ systems, including the nervous, endocrine and reproductive systems (e.g. epinephrine, corticosterone, opioids, testosterone, estrogen). Immune system activity can be broken down into innate and acquired (antigen-specific) immunity, and involves antigen processing, cellular, and humoral responses, all of which may play a role in autoimmunity.

Innate Immunity

The immune system is comprised of innate, nonspecific mechanisms, as well as acquired, or learned, antigen-specific mechanisms (Schedlowski & Tewes, 1999; Martino & Adorini, 1999). In contrast to acquired immunity, innate processes do not change with previous exposure to specific antigens. Thus, innate mechanisms function the same the first time that they encounter a foreign antigen, and each subsequent encounter. Innate immunity nonspecifically counters foreign antigens (e.g. bacteria, parasites, viruses) via anatomical and physiological barriers. The skin, mucusal lining the respiratory tract, and the microenvironment of the digestive system are all intended to keep foreign invaders out of the body. If an invader makes it past these anatomical barriers, other innate mechanisms nonspecifically launch the initial attack against the invader. Phagocytic cells, such as macrophages, neutrophils and monocytes are able to phagocytose foreign material and degrade it via hydrolytic enzymes. These cell types are attracted to foreign material by chemicals that are not specifically associated with a particular antigen. For example, chemotactic

responses in neutrophils and macrophages are initiated by methionine (a unique amino acid in peptides secreted by bacteria) and by interferons (IFNs) which are produced by virally infected cells (Schedlowski & Tewes, 1999; Martino & Adorini, 1999).

Antigen-Specific Immunity

While the innate immune system indiscriminantly responds to chemical signals released by a variety of cells, the acquired immune system is able to recognize and respond to specific antigens (e.g. a particular virus, bacteria type, or mutated protein from a tumor cell). This response is primarily mediated via T and B lymphocytes which are able to recognize antigen only if bound to the appropriate receptor. Antigen is processed and bound to major histocompatibility complex (MHC) receptors to be presented to T or B cells on the surface of antigen presenting cells (APCs) or abnormal cells (virally infected or tumor cells). Separate systems function for antigen that exists exogenously to the host cells, and antigen that exists endogenously, or within a host cell (Schedlowski & Tewes, 1999; Martino & Adorini, 1999).

Exogenous Antigen Processing. Macrophages, neutrophils, dendritic cells, and activated B cells are the only cells capable of presenting "exogenous" antigen, and are referred to as antigen presenting cells (APCs). They phagocytose foreign material (bacteria, parasites, fungi, etc.), degrade it via hydrolytic enzymes, bind the fragments to the second class of MHC receptors (MHC II), and present the foreign antigen on the surface of their cellular membrane in the context of the MHC II receptor. Only in the context of the

MHC II receptor can this antigen be recognized and responded to by T and B lymphocytes (Schedlowski & Tewes, 1999; Martino & Adorini, 1999).

Endogenous Antigen Processing. All nucleated cells are capable of expressing antigen in the context of MHC I receptors. Indeed a sampling of all of the proteins produced in a cell are presented on the surface of that cell in the context of MHC I receptors. After proteins have been transcribed in the endoplasmic reticulum, a subset are marked for degradation via enzymes, and are bound to MHC I receptors to be presented on the surface of the cell. Cells replicating viral DNA or RNA, or tumor cells producing abnormal proteins, will display these "non-self" proteins in their MHC receptors (Schedlowski & Tewes, 1999; Martino & Adorini, 1999). T and B lymphocytes must be able to distiguish between self and non-self in order to respond to only the foreign antigens (viruses and tumor cells). The immune system naturally develops a tolerance to all self antigen present before birth. The majority immune cells that respond to self antigen are selected and destroyed to keep proliferation and activity of these self-reactive cells to a minimum.

Thymus-Derived Lymphoctes. Immature lymphocytes are produced in the bone marrow. After maturation in the thymus (a primary lymphoid organ located above of the heart), various types of T cells emerge (Schedlowski & Tewes, 1999; Martino & Adorini, 1999). The thymus is a location where T cells can interact with various types of antigen, and where clonal selection occurs. Clonal selection involves the removal of T cells that react with self-antigens, or T cells that do not function properly. This immunological tolerance is necessary to

prevent the development of autoimmune diseases. T-cells consist of cytotoxic T cells (Tc), helper T cells (Th), and suppressor T cells (Ts). Each cell type has a very different function. Tc recognize antigen bound to MHC I, such as virally infected or tumor cells. They induce apoptosis in these cells by the release of cytotoxic factors such as perforins, granzymes, and lymphotoxins. Th cells facilitate other aspects of the cellular or humoral immune response. Ts cells are thought to be able to suppress various aspects of the immune response, though their very existence is controversial (Schedlowski & Tewes, 1999; Martino & Adorini, 1999).

B Lymphocytes. Humoral immunity is primarily composed of B cells, and their secretory product, antibodies, or immunoglobulin. When activated by the presence of antigen and a T helper cell, B cells divide and proliferate, producing memory cells and plasma cells. Memory cells exist for an extended period of time, for the purpose of quickening the second response to a particular antigen. Plasma cells are short lived secretors of immunoglobulin, or antibody. Secreted antibody can bind to antigen, to facilitate an immune response to that antigen (Schedlowski & Tewes, 1999; Martino & Adorini, 1999).

Natural Killer Cells. NK cells are derived from the same precursor cell as T lymphocytes, but differentiate outside the thymus. They are effective against virally infected cells, tumor cells, and some bacteria and fungi. Able to recognize antigen not in the presence of MHC receptors, they kill their targets via antibody dependent and independent mechanisms, similar to the Tc cells induction of apoptosis (Schedlowski & Tewes, 1999; Martino & Adorini, 1999).

T Helper Subclasses and Their Cytokines. T helper cells have two subclasses: Th1 cells promote cellular immunity, secreting pro-inflammatory cytokines (IFN-, IL-2, TNF-), Th2 cells promote humoral immunity and secrete primarily anti-inflammatory cytokines (IL-4, IL-10, IL-13) (Elenkov & Chrousos, 1999). See Figure 1 for illustration (Elenkov & Chrousos, 1999). Bipotential precursors of Th1 and Th2 cells, Th0 cells, are influenced by cytokines produced by the innate immune system to differentiate into Th1 or Th2 cells. Monocyte/macrophage produced IL-1, and NK-cell-derived IFN-, work in concert to induce Th1 differentiation. The activated Th1 cell secreted IFN-, along with the APC-derived IL-12 and TNF-, stimulate the activity of Tc, NK cells, and activated macrophages (the primary components of cellular immunity). In contrast, IL-4 and IL-10 promote humoral immunity by stimulating the differentiation of antibody-secreting B cells, the growth and activation of mast cells, and eosinophils, and the immunoglobulin switching to IgE. Th1 and Th2 responses are mutually inhibitory, with IL-12 and IFN- inhibiting Th2 responses, and IL-4 and IL-10 inhibiting T cell proliferation, macrophage activation, and Th1 cytokine production (Elenkov & Chrousos, 1999).

Potential Causes of Autoimmunity

Autoimmune processes are not uncommon in the normal functioning of the immune system, and are essential to the removal of aged or damaged cells (Schedlowski & Tewes, 1999; Martino & Adorini, 1999). Normal individuals possess self-reactive lymphocytes in small numbers that are typically suppressed by immune regulatory mechanisms. Though the exact cause of

autoimmune diseases remains unknown, many hypotheses of what triggers the pathogenic development exist: the formation of new epitopes (portions of a molecule that are immunoreactive) on normal cells, the exposure of hidden

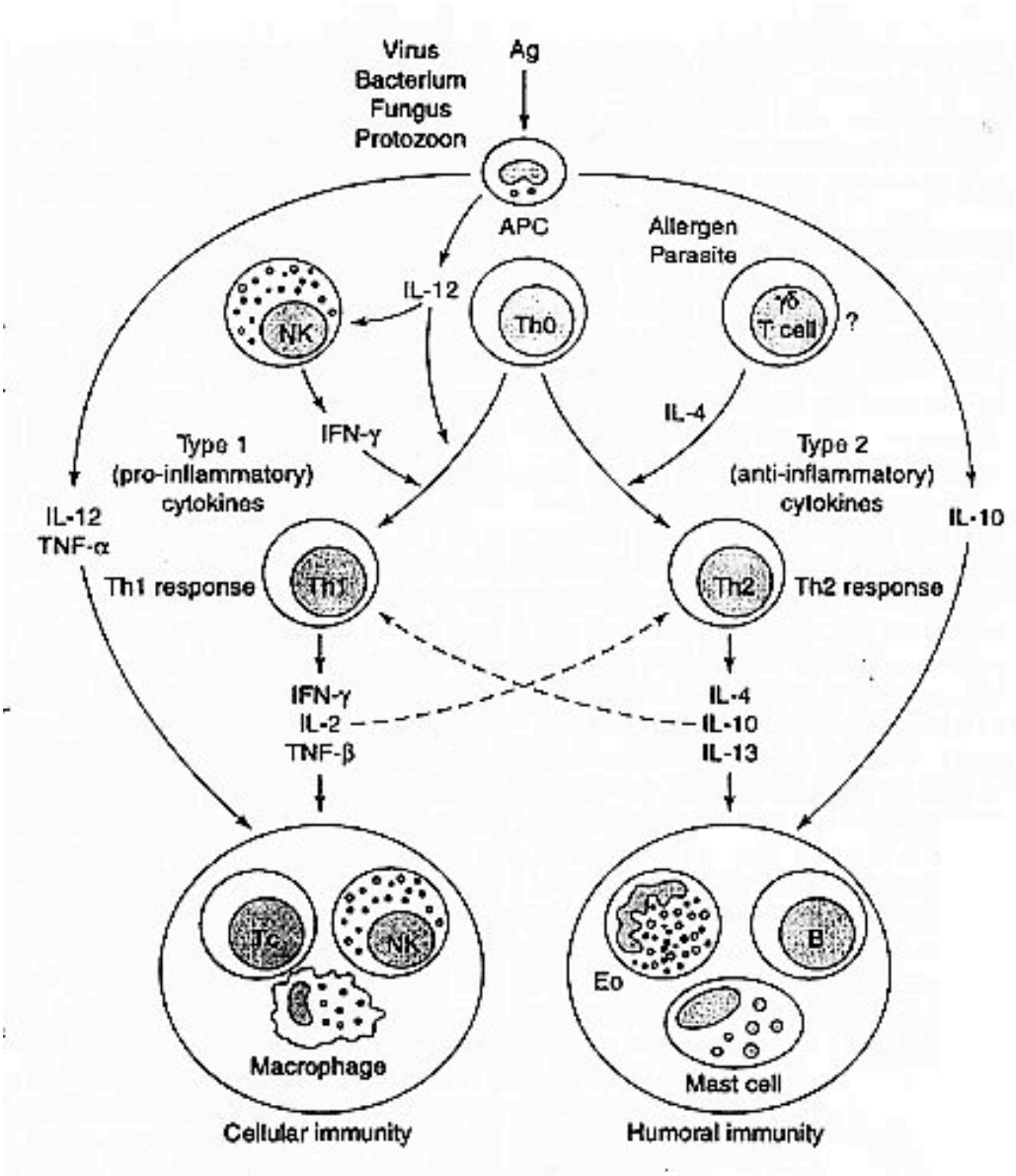

Figure 1. Th1 and Th2 Immunity. (Elenkov & Chrousus,1999).

molecules on or inside normal cells, viral infection, molecular mimicry between foreign epitopes and self-epitopes, and the loss of control of lymphocyte responses. When immunoglobulin molecules bind to antigen, a different portion of the Ig molecule is exposed, forming a new epitope that self-lymphocytes recognize as foreign. Rheumatoid factors (antibodies that respond to self-antibodies) are involved in the autoimmune diseases Rheumatoid Arthritis and Systemic Lupus Erythromyelitis, where large quantities of these immune complexes exist. Another hypothesis involves cells that are not typically exposed to the immune system, such as those found in testicular and nervous tissue, and intracellular molecules, which are not tolerated by the immune system. Damage to the testes or CNS, and the release of intracellular molecules, exposes these hidden antigens to the immune system, causing an immune response to be mounted against them. Viral infection is also thought to be a triggering event for various autoimmune diseases. Viral destruction of tissue results in an immune mediated effort to remove the dead or damaged cells. Another possible mechanism involves viral infection of lymphoid tissue, which can interfere with immunological control mechanisms. Molecular mimicry is a hypothesis that involves foreign epitopes from bacteria, parasites, etc. with a similar structure and sequence to self-epitopes. Once an immune response is mounted against the foreign epitope, the self-epitope is mistaken as foreign as well. Of these alternatives, the most probable cause of autoimmunity is the loss of regulatory mechanisms within the immune system that hold the low levels of self-reactive lymphocytes in check. The loss of regulation of these cells can lead

to increased proliferation and pathology (Schedlowski & Tewes, 1999; Martino & Adorini, 1999).

Overview of the Stress Systems

Some of the major regulatory devices that hold the immune system in check are the stress systems. The physiological systems that respond to stress include the hypothalamic-pituitary-adrenal axis and the sympathetic branch of the autonomic nervous system, which is activated by arousal systems in the brain (Chrousos et al., 1998). When homeostasis is threatened by an internal or external physical or psychological stressor, these systems are engaged to help ensure the survival of the individual and the species. Energy must be conserved from less immediately necessary behaviors, such as feeding, growth, reproduction, and to an extent - some functions of the immune system. This energy can then be utilized for mobilization and adaptive defensive behaviors. The stress systems initiate a repertoire of physiological responses and behaviors that are designed to accomplish this. See Figure 2 for an overall illustration of this.

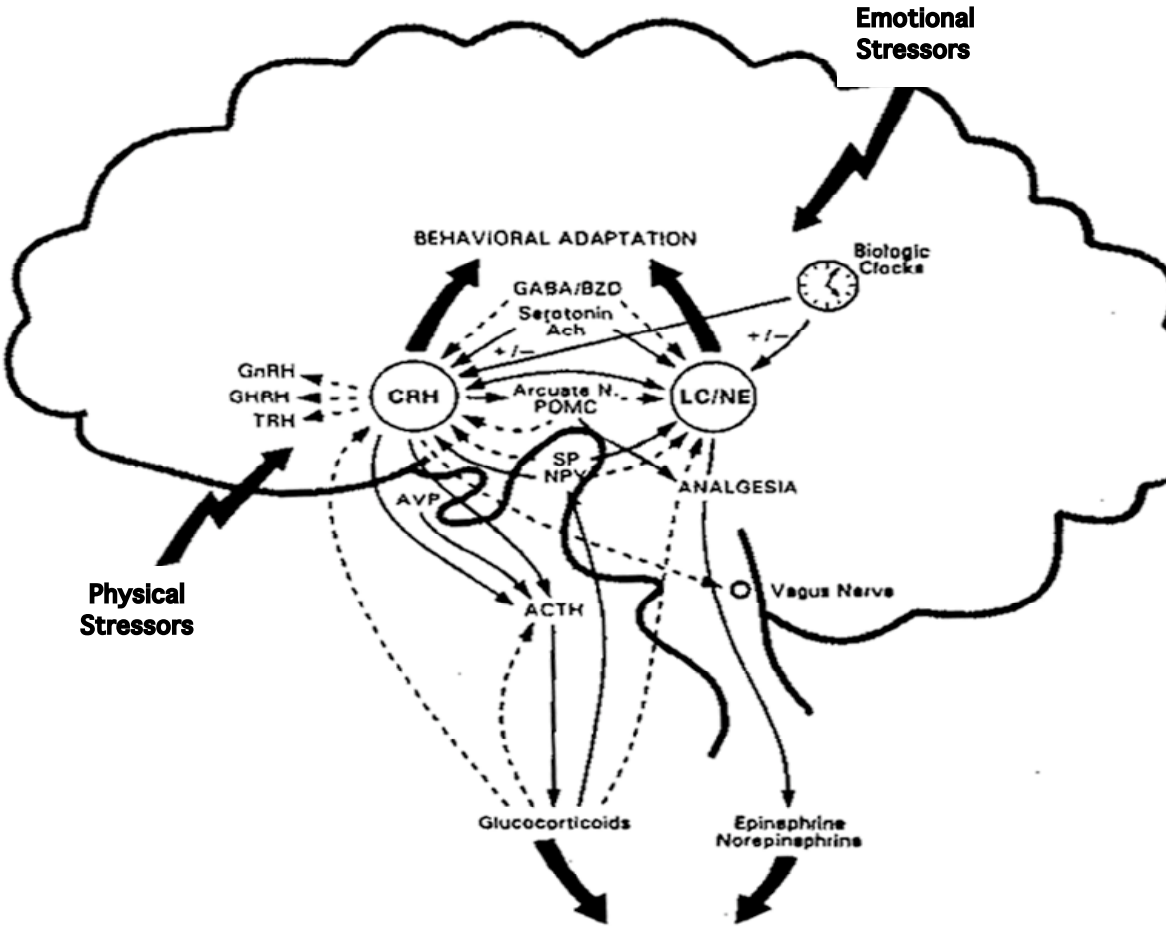

Figure 2. Physiological Systems That Respond to Stress (Adapted from Stratakis & Chrousos,1997).

Hypothalamic Pituitary Adrenal Axis (HPA)

Corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) are secreted by the parvocellular neurons of the paraventricular nucleus of the hypothalamus into the hypophyseal portal system (Chrousos et al., 1998). These molecules are the primary modulators of the HPA axis. CRH induces and is permissive to the secretion of adrenocorticotropin hormone (ACTH) from the pituitary into the general circulation. AVP, while acting synergistically with CRH to cause the release of ACTH, has little or no effect on the secretion of ACTH on its own. ACTH reaches the adrenal glands, which rest above the kidneys, and induces the secretion of corticosterone (CORT) from the adrenal cortex. CORT, in turn, has wide ranging effects on multiple organ systems, including the immune and reproductive systems, as well as a negative feedback effect on ACTH and CRH production (Chrousos et al., 1998).

Sympathetic Branch of the Autonomic Nervous System

The locus coeruleus is a noradrenergic brain stem nucleus that regulates arousal of the central nervous system, as well as the sympathetic branch of the autonomic nervous system (Chrousos et al., 1998). The acute response to stress is mediated by the rapidly responding sympathetic nervous system through innervation of many organs, including the gut, kidney, and adrenal medulla (Stratakis & Chrousos, 1997). The sympathetic and parasympathetic nervous systems have the capability of releasing acetylcholine, norepinephrine, epinephrine, neuropeptide Y (NPY), somatostatin, enkephalin, and a variety of

other neuropeptides. The secretion of these substances is modulated by the HPA axis.

Interactions Between the HPA Axis and SNS

Similar neuroendocrine substances activate or inhibit the CRH producing neurons of the hypothalamus and the noradrenergic neurons of the locus coeruleus. Serotonin and acetylcholine stimulate these structures, while GABA/benzodiazepines, ACTH, and glucocorticoids cause inhibition (Stratakis & Chrousos, 1997). The paraventricular nucleus and locus coeruleus are also able to modulate each other via reciprocal neural connections. Additional negative feedback loops exist between the two systems. Glucocorticoids released from the HPA axis are able to suppress the activity of the noradrenergic system, as well as CRH and ACTH production.

A Balance Between the Neuroendocrine and Immune Systems

The neuroendocrine systems that respond to stress are critical modulators of the immune system (Sternberg, 1995). Communication between the immune and neuroendocrine systems during an inflammatory response can determine if the actions of the immune system prove beneficial or detrimental to the overall health of the animal. Chemicals released by the immune system during an inflammatory response (cytokines) activate the HPA axis, causing the release of corticosteroids which provide a negative feedback mechanism, suppressing the ongoing immune response. Disruption of this negative feedback loop between the immune and stress systems can lead to a hyper-

responsive inflammatory response, and potentially, autoimmune conditions. See Figure 3 from Sternberg (1995) for an illustration of this loop.

Glucocorticoids and catecholamines are known to profoundly effect the functioning of the immune system and some research has focused on the effects of the modulation of these hormones experimentally on autoimmunity

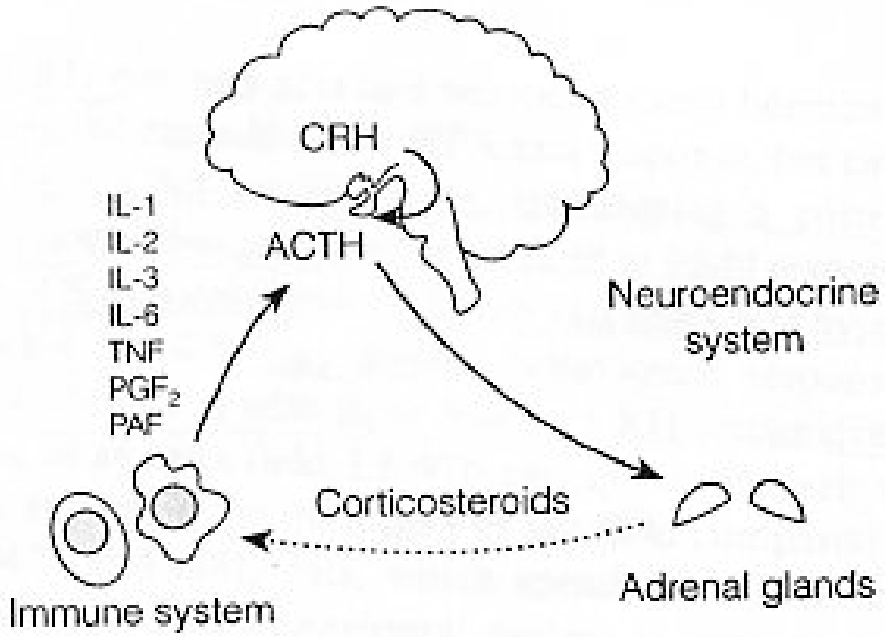

Figure 3. Bidirectional Communication Between the Immune and Neuroendocrine Sytsems (Sternberg,1995).

Pharmacological doses of adrenal steroids are a commonly used treatment of inflammatory and autoimmune conditions (McEwen et al., 1997). Glucocorticoids are able to suppress Th1 cells and alter the trafficking of

immune cells to the site of infection (McEwen et al., 1997). Epinephrine and NE are released by the adrenal glands (following pituitary hormone stimulation) and the nerves of the autonomic nervous system (Chelmicka Schorr & Arnason, 1999). Sympathetic innervation of the lymphoid organs, lymph nodes, spleen, thymus, gut-associated lymphoid tissue allow for contact with T cells, monocytes and B cells. β_2-adrenergic receptors exist on lymphocytes (B cells, NK cells, Ts cells, Tc cels, Th cells) and macrophages.

Stress and Immunity: Factors to Consider

The experience of stress can lead to a transient increase in the activation of the stress systems, which subsequently alters immune system functioning. However, *how* psychological stress affects the course of autoimmunity is a complex picture, and is determined by a multitude of factors. The duration, severity, and type of the stressor can produce different patterns of activation in the stress systems, which in turn would differentially modulate immune processes (Elenkov & Chrousos, 1999). Dhabhar and colleagues (1997) discovered different effects of the same stressor on cellular immunity dependent on the *duration* of that stressor. One 2-5 hour session of restraint stress increased skin delayed-type hypersensitivity reactions and leukocyte deployment to the skin. However, chronic exposure to restraint stress (daily sessions for 3-5 weeks) caused a marked decrease in both of these measures. Similarly, McEwen and colleagues (1997) found divergent effects of the same type of stressor dependent on the *severity* of the stressor. Exposure to brief, mild tailshocks

increased NK cell activity and lymphocyte IL-2 production, while exposure to more prolonged intense shock dramatically decreased increased NK cell activity and lymphocyte IL-2 production. Though chronic restraint stress causes an overall immunosuppression, another *type* of stressor, social disruption, has a very different effect. Sheridan and colleagues (Avitsur, Stark & Sheridan , 2001; Stark et al., 2001), have developed a stressor that disrupts the social hierarchy of young male rodents by the introducing a dominant, older, aggressive, male conspecific. Following chronic administration of this stressor (6 sessions over 7 days), animals display an insensitivity to the antiproliferative effects of corticosterone in splenic lymphocytes (primarily macrophages). This glucocorticoid resistance has been found to cause an augmentation of the inflammatory and antiviral immune response to influenza A (Avitsur et al., 2001; Sheridan, Stark, Avitsur, & Padgett, 2000). In essence, the lack of the negative feedback effect of glucocorticoids on the immune response allowed the inflammatory response to influenza to go unchecked. This has obvious implications for autoimmune diseases. In addition to the transient effects of stress, prolonged activation of the stress systems can lead to a change in the future functioning of the stress systems themselves. It is theorized (McEwen, 1998) that frequent stress and activation of the stress systems could lead to a rebound effect, where the systems habituate and no longer appropriately respond to immune and stress challenges.

To further complicate the picture, the same stressor can alter various aspects of the immune response differentially. Activation of the stress systems

has been found to suppress cellular immunity, while enhancing humoral immunity (Elenkov & Chrousos, 1999). This results from a shift in the balance between Th1 (pro-inflammatory) and Th2 (anti-inflammatory) cytokines. Glucocorticoids reduce the production of IL-12 (the main inducer of Th1 cells) by APCs, and monocytes/macrophages, and downregulate IL-12 receptors on T and NK cells. As a result, T cells produce less IFN-γ and more IL-4. In addition, monocyte production of IL-10 is increased. Catecholamines likewise decrease Th1 immunity, while increasing Th2 immunity (Elenkov & Chrousos, 1999). Stimulation of β-adrenergic receptors by NE or epinephrine enhances IL-10 production, while suppressing IL-12 production. β_2- adrenergic receptors exist on only Th1 cells, but not Th2. In general, CORT and catecholamines suppress cellular immunity, while enhancing humoral immunity. This data should caution us against using one immune measure to generalize about the effects of stress on the entire immune system, the organism, or disease process. It is important to look into the abundance of a given immune component, as compared to other immune components, as well as the distribution throughout the body of that immune component, and the functioning of that component. Stressors can not only alter the number of various immune cells in a particular area (e.g. blood, spleen), but can also alter their activity, proliferation, cytokine production, and receptor expression when stimulated (McEwen et al., 1997). What parameter is measured (type of immune cell or secretory product), how it

Table 1. Factors to consider when investigating the effects of stress on autoimmunity.

	Factors To Consider	**Examples**
Stressor	Type	restraint, social disruption
	Severity	1mA or 3mA tailshock
	Duration	15 min, 12 hours
	Chronicity	one exposure, daily exposure
Immune Component	Type	cellular, humoral, innate
	overall amount	# of lymphocytes
	distribution throughout the body	immune cells in blood, spleen, skin
	degree of functioning	proliferation to mitogen
	in proportion to other components	TH1/TH2 balance
Autoimmune Disease	phase of the disease (timing)	prior to, during
	mechanism of the disease	T-cell or B-cell mediated

Table 2. Divergent effects of stress on immunity.

Category of Stress	**Effects of Stress**
Mild-Moderate, Acute	immune enhancement
Severe, Acute	immune suppression
Chronic	generally immune suppression
Following Chronic	habituation of stress response glucocorticoid resistance

is measured (number of cells vs. activity of cells), and where it is measured (general circulation, immune tissues, site of infection or wounding) could determine if an immunoenhancing, or suppressing effect is found (see Tables 1 and 2 for summary).

Stress and Immunity: Summary and Conclusions

Obviously a link between stress and autoimmunity exists, but determining the exact nature of that relationship is a complex issue. The stress systems play an important role in regulating the immune response. Without the appropriate negative feedback loop in place, animals, and potentially human beings, are at risk of developing autoimmune conditions. Exposure to psychological stress may be a means by which to disrupt the process of regulating the immune system via stress hormones. Stress can shift the immune response to a more Th1 cellular immunity, proving detrimental to the course of most autoimmune diseases. It can also produce glucocorticoid resistance, eliminating the stress system's ability to interact with the immune system. Prolonged exposure to stress could cause a habituation of the stress response, leading to the same outcome. Though a powerful force in modulating the immune responses in autoimmune conditions, the type, duration, severity, and frequency of the stressor must be taken into consideration before making any predictions for disease outcome. Likewise, the time point at which the stress occurs within the autoimmune disease, as well as the mechanism of the disease process prove to be important predictive factors.

Sex and Sex Hormones

Major influences on the functioning of not only the immune system, but also the stress systems, are sex and sex hormones. It is widely known that the incidence of autoimmune disease is far greater in women than men, ranging from 2-3:1 to 25-50:1(Homo-Delarche et al., 1991; Whitacre et al., 1999). Though the reason behind this disparity is still under investigation, sexual dimorphism of the immune system, regulation of the immune system and autoimmune diseases by sex hormones, and differences in the functioning of the stress systems across gender have been implicated.

Sexual Dimorphism of the Immune System

The immunological environment of males differs greatly from that of females, in ways that are of specific relevance to autoimmune diseases. In general, females have a more pronounced immune response, with greater primary and secondary immune responses, higher levels of serum immunoglobulins, greater and more prolonged antibody responses, and increased cell-mediated immunity (Homo-Delarche et al., 1991; Gaillard & Spindedi, 1998; Whitacre et al., 1999). Women possess a more developed thymus, have higher rates of rejection of tumors and homografts, and have a greater resistance to the induction of immunological tolerance (Homo-Delarche et al., 1991).

This sexual dimorphism of the immune system may in part be due to modulation via sex hormones. The homeostatic control that the endocrine system has over the immune system is represented not only in the dramatic

effects of stress hormones such as corticosterone, epinephrine on autoimmune phenomena, but also in the regulation of immune processes by sex hormones, such as estrogen, progesterone, and testosterone. The effect of estrogen on autoimmune diseases varies greatly (as will be discussed further below), while the effects of androgens tend to be universally protective against autoimmunity (Homo-Delarche et al., 1991).

The hypothalamus secretes gonadotropin-releasing hormone (GnRH) which causes the anterior pituitary to secrete follicle stimulating hormone (FSH) and leutinizing hormone (LH) into the general circulation. LH stimulates the Leydig cells of the testes to secrete testosterone, some of which gets converted to DHT (dihydrotestosterone). These androgens may decrease antibody production by activating suppressor T cells. They have been found to increase CD8+ T cells (suppressor/cytotoxic) in mouse spleen and lymph nodes, and can dose-dependently inhibit the proliferation of human lymphocytes. In birds, androgens directly inhibit the development of the *Bursa of Fabricius*, which would down-regulate B cell differentiation and thus antibody production.

The hypothalamic-pituitary-ovarian axis controls the female reproductive system (Chrousos et al., 1998). GnRH is secreted by the hypothalamus (arcuate and preoptic neurons) into the hypophyseal portal system, which in turn causes the pituitary to secrete FSH and LH into the general circulation. These hormones induce the ovaries to secrete estrogen and progesterone, which act on a multitude of reproductive organs and other tissues, including the immune system (Chrousos et al., 1998). Estrogens have been found to regulate monocyte

and lymphocyte numbers, decreasing CD8+ T cells (suppressor/cytotoxic) in the spleen, thymus, and lymph nodes, and increasing B cell differentiation and antibody production (Homo-Delarche et al., 1991; Luster, Pfeifer, & Tucker, 1985). These effects contrast that of androgens which overall tend to be immunosuppressive.

Sex hormones have an important role in autoimmune diseases, but exactly what that role is depends on the immunopathological mechanism of the specific autoimmune disease (Whitacre et al., 1999). Th2, or antibody-mediated diseases, involve B cell activation and/or circulating immune complexes. Th2 lymphocytes secrete anti-inflammatory cytokines: interleukin (IL)-10, IL-4, IL-5, and IL-6 (Kim et al., 1999). In contrast, cell-mediated diseases are alternatively a Th1, or, with pathology primarily revolving around T cell mechanisms. Th1 cells alternatively secrete interferon gamma (IFN-γ) and tumor necrosis factor beta (TNF-β) (Kim et al., 1999). Important when considering the role of estrogen, is it's biphasic effects on Th1 and Th2 dependent immunity. Testosterone, progesterone, pregnancy, and *high* levels of estrogen increase the Th2 mediated response while suppressing the emergence of the Th1 cells. In contrast, prolactin and *low* levels of estrogen lead to a more Th1 mediated response (see Figure 4 adapted from Whitacre et al., 1999). Whether a given disease is Th1 or Th2 dependent would predict if specific sex hormones would alleviate or exacerbate the symptoms of the disease.

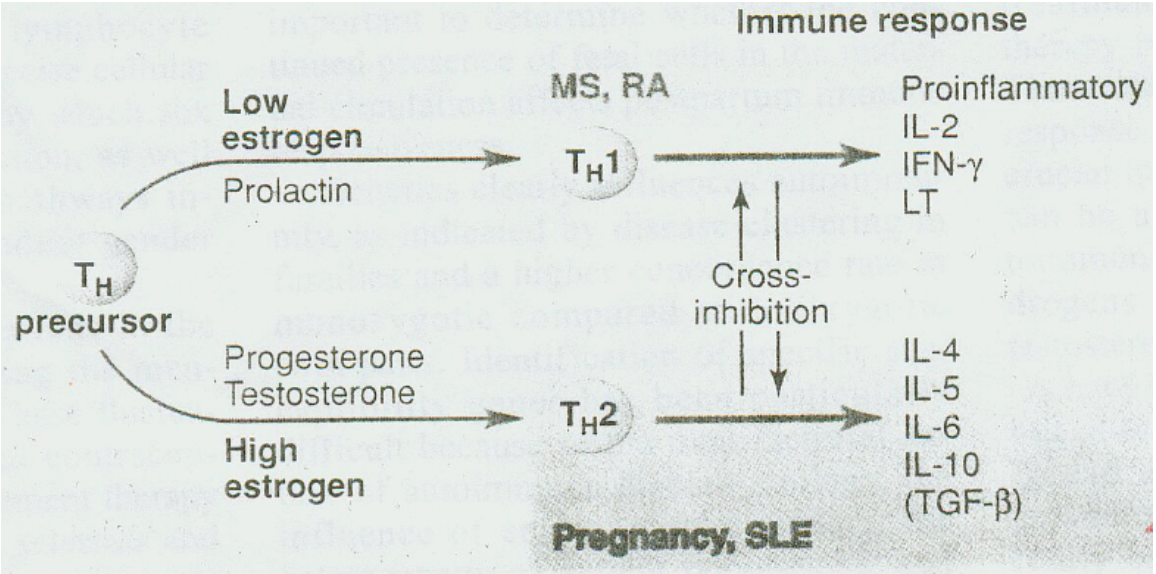

Figure 4. The Determination of Th Subclass by Sex Hormones. (Whitacre, Reingold, & O'Looney, 1999).

Sexual Dimorphism of the Stress Response

In addition to a sexually dimorphic immune response, males and females also differ in the functioning of their HPA axis (Gaillard & Spinedi, 1998). Higher basal levels and a higher diurnal rise in plasma CORT are observed in female rats, with higher corticosteroidgenesis by adrenal slices in vitro (Homo-Delarche et al., 1991; Gaillard & Spinedi, 1998; Turner, 1990). This hypercortisolism is also seen in women and could be partially due to the activation of the CRH promotor gene and noradrenergic system by estrogen (Chrousos et al., 1998). Estradiol has been found to stimulate CRH synthesis, stimulate cortisol-binding globulin secretion, and potentiate the actions of norepinephrine (Chrousos et al., 1998). Indeed, the HPA axis has been found to

be most sensitive to stress during proestrus, suggesting a facilitory effect of estrogens (Homo-Delarche et al., 1991; Gaillard & Spinedi, 1998; Viau & Meaney, 1991). The hyper-responsiveness of the HPA axis following estrogen administration can also be seen in normal men (Chrousos et al., 1998).

In response to stress or ACTH, female rats also show a greater and more prolonged elevation in plasma CORT. This may be linked to the lower levels of pituitary glucocorticoid receptors (GCRs) in female rats, which is estrogen dependent (Homo-Delarche et al., 1991). Estradiol has been found to result in reduced GCR binding in the anterior pituitary, hypothalamus, and hippocampus, which would in turn depress the negative feedback effects of CORT, and would lead to increased HPA axis activity (Chrousos et al., 1998). Akin to the pattern seen with rats, in the NOD mouse, glucocorticoid levels were found to be higher in females under basal conditions, and during immobilization stress females were unable to adapt, while their male counterparts did (Homo-Delarche et al., 1991). In contrast to the effects of estrogen on the HPA axis, an inverse correlation between testosterone levels and HPA axis activation has been found throughout the development of male mice (Gaillard & Spinedi, 1998).

Increased levels of CRH and CORT in females are not necessarily due to the effects of sex hormones on the HPA axis. Immune tissues (immune organs and inflammatory sites) and reproductive tissues (ovarian, testicular, endometrial, and placental) are also capable of secreting CRH, and ultimately causing the release of corticosterone from the adrenals. In fact, placental

secretion of CRH during the later half of pregnancy explains the hypercortisolism observed at this time (Chrousos et al., 1998). Many autoimmune diseases (Th 1 mediated) are known to be less severe during pregnancy, followed by a possible increase in symptomatology of these diseases post-partum. Withdrawal from placental CRH causes a secondary hypothalamic CRH deficiency (Chrousos et al., 1998).

Beyond the effects of CRH and corticosterone, neurotransmitter levels in certain brain regions have been found to be sexually differentiated and under the influence of sex steroids. Women are known to have a greater incidence of depression, which may be linked to differences in serotonin levels in the brain. Indeed, a sex-related disparity in serotonin levels in rat brain has been discovered. More interestingly, in humans there appears to be a sex difference in the central serotonergic regulation of prolactin, ACTH, and cortisol (Homo-Delarche et al., 1991; Kennett, Chaouloff, Marcou, & Curzon, 1986; Maes et al., 1989).

Effects of Stress on Sex Hormones

In general, the HPA axis has a suppressive effect of both female and male reproductive systems, while the sympathetic nervous system has a facilitory effect (Chrousos et al., 1998; Homo-Delarche et al., 1991). Corticotropin-releasing hormone (CRH) inhibits gonadotropin-releasing hormone (GnRH), which would in turn suppress the release of FSH and LH, subsequently reducing the release of estrogen and progesterone (Chrousos et al., 1998). Cortisol acts at all levels of this system: hypothalamus, pituitary, ovaries and

target tissues for gonadal secretions. CORT inhibits GnRH secretion, LH secretion, estrogen and progesterone biosynthesis, and the effects of estrogen on target tissues (Chrousos et al., 1998). A common example of the effects of stress on the female reproductive system is the functional hypothalamic amenorrhea, which can be induced in up to 100% of women in severely stressful situations (e.g. prisoners before execution). The secretion of testosterone from the testis is likewise suppressed by the actions of corticosterone (Homo-Delarche et al., 1991). In humans, physical exercise, intense mental stress, and critical illness have all been shown to lead to a decrease in testosterone concommitant with an increase in CORT levels. In rodents, immobilization stress has resulted in reduced testicular testosterone content (Homo-Delarche et al., 1991; Harkonen, Naveri, Kuoppasalami, & Huhtaniemi, 1990; Christeff, Benassayag, Carli-Vielle, & Nunez, 1988; Charpenet et al., 1981; Armario & Castellanos, 1984; Sapolsky, 1985; Mann & Orr, 1990).

Norepinephrine released by the locus coeruleus/sympathetic nervous system, in contrast, stimulates the release of GnRH (Chrousos et al., 1998). This effect however is often completely countered by the concurrent activation of the HPA axis (Chrousos et al., 1998). See Table 3 for summary.

Table 3. Stress induced changes in sex hormone production.

Hormone	**HPA effects**	**LC/SNS effects**
GnRH	decrease	Increase
LH	decrease	countered by HPA
FSH	decrease	countered by HPA
Estradiol	decrease	countered by HPA
Progesterone	decrease	countered by HPA
Testosterone	decrease	countered by HPA

Sex, Stress, and Autoimmunity: Summary and Conclusions

There is a preponderance of autoimmune diseases in women as compared to men, ranging from 25-50:1 to 2-3:1. Sexual dimorphism of the immune system, the actions of sex steroids, and sexual dimorphism of the HPA axis and noradrenergic systems may all contribute to this disparity. Overall, women tend to have a more pronounced cellular and humoral immune response than men. A bi-directional communication exists between the HPA and HPG axes, both of which can dramatically effect the functioning of the immune system. In general, estrogens activate the HPA axis, while products of the HPA axis suppress the female and male reproductive systems. Increased HPA axis activation, androgens, and estrogens have a tendency to help alleviate autoimmune disorders. However, the biphasic effects of estrogen must be taken into consideration. Low levels of estrogen lead to a Th1 immune response, perfect for the development of most autoimmune diseases which are Th1

mediated. In contrast, high levels of estrogen seen in pregnancy and the use or oral contraceptives lead to a more Th2, or humoral-mediated immunity, which exacerbates the symptoms of SLE (a Th2 mediated disease), while suppressing the other Th1, or cell-mediated autoimmune diseases. Additionally, it has been suggested that it is the androgen/estrogen ratio that renders people susceptible to autoimmune diseases. According to this theory it would not be the level of estrogen per se, but the relative androgen deficiency that would enhance autoimmune phenomena.

Stress and Multiple Sclerosis

Multiple Sclerosis Background

Multiple Sclerosis (MS) is one of the most common demyelinating conditions of the central nervous system (CNS), affecting 350,000 people in the United States alone (Anderson et al., 1992). Eighty percent on MS patients have the relapsing-remitting form of MS, involving symptomatology evolution over a period of days followed by stabilization and improvement (Noseworthy et al., 2000). This form may turn into secondary progressive if the disease progresses between relapses. The other 20% of MS patients have primary progressive MS, which has a gradually progressive clinical course. There is a 2:1 female predominance of relapsing remitting MS, but the incidence of primary progressive MS is similar among men and women. In contrast to the female predominance in the incidence of MS, males tend to have a more severe clinical course. Symptoms include sensory disturbances, optic neuritis, limb weakness,

clumsiness, gait ataxia, neurologic bladder and bowel symptoms, as well as some cognitive impairments. MS is characterized by infiltration of inflammatory cells, focal demyelination, relative preservation of axons, and astrocytic scars in the CNS (Noseworthy et al., 2000). Inflammatory cells are generally perivascular, but may diffuse into the parenchyma, and are typically composed of lymphocytes and macrophages. Early symptoms of MS are thought to be due to axonal demyelination, which slows nerve conduction. However, inflammatory edema, inflammatory cytokines, irreversible axonal injury, and gliotic scarring may also be involved (Noseworthy et al., 2000). The etiology of MS remains unknown, but both genetic and environmental factors have been implicated. Twin studies have found the concordance rate among monozygotic twins to be 31% compared to the 5% concordance rate in dizygotic twins (Sadovnick et al., 1993). Family studies have found the risk of MS for a first-degree relative of a patient to be 20-40 times the risk in the general population (Sadovnick et al., 1988). Disease susceptibility has also been linked to the HLA region on chromosome six (Jersild et al., 1973). In addition to genetic susceptibility, exposure to a pathogen has proven to be a likely determining factor in the development of MS (Noseworthy et al., 2000). Epidemiological studies have found the susceptibility rate of MS to be linked to geographical location, but migration to an area with a different rate of MS (before adulthood) changes this susceptibility (Kurtzke, 1991). Even more compelling, in the Faroe islands there has been evidence of outbreaks of MS, or clustering of cases in terms of geography and time (Kurtzke & Hyllested, 1987).

Isolation of many pathogens to a greater degree from MS tissue than control tissue has implicated them in the onset of MS, including human herpes virus type 6 and *Chlamydia pneumoniae* (Noseworthy et al., 2000). Additionally, MS patients are advised to limit exposure to viral illnesses because infections may trigger relapses (Sibley et al., 1985). Thus, a combination of genetic susceptibility and exposure to a pathogen may be the trigger for developing MS. Treatment of MS includes immunomodulators such as interferon beta and immune globulin. Interestingly, one of the most effective treatments of relapses of MS is administration of corticosteroids (Noseworthy et al., 2000).

While it is difficult to obtain experimental evidence of the effects of stress on MS in humans, it is widely recognized that MS patients report periods of stress prior to the onset of the disease and before exacerbations of their symptoms. A recent meta-analysis of 14 studies investigating stress and MS that were published from 1965-2003 found a significant increase in the risk of disease exacerbation following a stressful life event (Mohr et al., 2004). The average weighted effect size for stress was d= 0.53 (Mohr et al., 2004), while the effect size for the principle class of disease modifying drugs used to treat MS, interferon beta, ranged from d= 0.36 to 0.30 (Filipini et al., 2003). Based on this, it could be concluded that stress has a greater impact on MS symptomatology than the current drugs available to treat MS.

As early as 1877, Charcot reported that stressful experiences precipitated the onset of MS (Charcot, 1877). Mohr and Cox (2001) reviewed the effects of stress on multiple sclerosis. Many studies since Charcot's report have found

that MS patients, as compared to healthy controls, or patients with other neurological disorders, report more stressful experiences prior to initial symptomatology. Additionally, longitudinal studies find stress to increase the chances of exacerbation of MS (Mohr & Cox, 2001; Ackerman et al., 2003). The type of stress, however, may be important. While relatively moderate and chronic stressors seem to follow the pattern of stress-induced exacerbation of disease, severe stressors (e.g., war, see Nisipeanu & Korczyn, 1993) have been found to lower the rate of relapses of MS. Nisipeanu and Korczn (1993) utilized an Israeli population of MS patients that had been under investigation prior to and following missile attacks during the Persian Gulf War. These patients reported significantly fewer relapses during this stressful period than any other period of time under investigation. The type or severity of the stressor in this instance may be the determining factor. Mohr et al. (2000) found less severe stressors such as increased conflict, disruption of routine, and daily hassles to predict the risk of developing new brain lesions 8 weeks later, while there was no effect of severe stress on brain lesion development. Yet, Ackerman et al. (2003) found exacerbations in MS symptoms to be more likely following stressful life events, *independent* of type of stressor. Because the relationship between stress and MS in humans is complex, this research domain is likely to benefit from the investigation of stress using animal models.

Animal Models of MS

The use of animal models of MS offers the possibility of investigating the effects of stress in controlled experiments. This data is important for

understanding and explaining the correlational relationship between stress and MS found in the human literature. The two most common animal models used to study MS are Experimental Allergic Encephalomyelitis and Theiler's Virus Induced Demyelination.

Experimental Allergic Encephalomyelitis (EAE). EAE is an experimentally induced model, where homogenized CNS tissue or myelin components are injected subcutaneously into the animal in combination with an immune stimulator, Complete Freund's Adjuvant (CFA). The introduction of the myelin antigen with the peripheral immune stimulation induces an autoreactive immune response directed at the animal's own myelin. Once autoreactive T cells are generated, the transfer of these T cells to another host can also induce EAE. The resulting inflammatory demyelination of the spinal cord causes weakness in the limbs, motor impairment, and eventually paralysis and incontinence. The mechanism of EAE pathogenesis revolves around the induction of the autoreactive T cells. Stimulated and activated CD4+ T cells increase adhesion molecules, enter the CNS, and secrete proinflammatory Th1 cytokines, leading to the recruitment of mononuclear cells. B cell secretion of anti-myelin antibody (Ab), in concert with macrophage/glia secreted cytotoxic factors, leads to demyelination (Tsunoda and Fujinami, 1996). In this exclusively autoimmune mediated model, suppression of the immune system leading to an alleviation of the disease process would be an expected result.

Theiler's Virus Induced Demyelination (TVID). Theiler's virus induced demyelination (TVID), though similar to EAE in the resulting demyelination,

behavioral signs of chronic disease, and histological lesions, involves a different immunopathological pathway (for reviews see: Dal Canto et al., 1995; Tsunoda and Fujimani, 1996; Oleszak et al., 2004). Theiler's Murine Encephalomyelitis Virus (TMEV) is a naturally occurring pathogen that has been isolated from mice. In the early stages of infection the virus causes an asymptomatic central nervous system infection. In susceptible strains of mice that cannot clear the virus from their CNS, a persistent inflammatory demyelination ensues (Welsh, Blakemore, Tonks, Borrow, & Nash, 1989). In the first few weeks following innoculation with a live TO strain of Theiler's virus, CNS neurons are infected with the virus. During this acute phase of the infection, Theiler's virus specific cellular and humoral immunity removes the virus from the gray matter, inducing apoptosis of the virally infected neurons. Though typically asymptomatic, under conditions of immunosuppression, mice may display symptoms of encephalitis (Campbell et al., 2001). Following this acute phase of Theiler's virus infection, viral infection persists in glia and macrophages located in the white matter throughout the chronic phase of the disease when demyelination and paralysis occur (Brahic et al., 1981). During this phase, additional macrophages, Theiler's virus specific T cells and antibody are recruited to the CNS, inducing oligodendrocyte apoptosis, inflammation, and demyelination (Tsunoda & Fujinami, 1997). Multiple mechanisms of demyelination have been found in TVID, including: direct viral lysis of oligodendrocytes (Roos and Wollmann, 1984), bystander demyelination mediated by virus specific DTH T cells (Clatch et al., 1987), cytotoxic T cell

reactivity (Rodriguez and Sriram, 1988), and autoimmune mediated demyelination (Welsh et al., 1987; Miller et al., 1997; Borrow et al., 1998). Following the immune response directed against Theiler's virus, epitope spreading leads to CD4+ T cells reactive to myelin components (Miller et al., 1997; Borrow et al., 1998; Miller et al., 1997). Virus-specific T cell responses cause bystander destruction of myelin. Recruited and CNS-resident antigen presenting cells process and present these endogenous myelin epitopes to autoreactive T cells (Miller et al., 1997). Antibody to myelin is also present at this later stage of the disease (Welsh, et al., 1987).

Stress and EAE. In contrast to stress exacerbating the development MS, most studies find general pattern of alleviation in the animal model of MS, EAE, when exposed to various stressors. Bukilica and colleagues (1991) looked at the effects of 19 daily sessions of inescapeable tail-shock (80, 5 s, 1 mA) or noise stress (60, 5 s, 90 dB fire alarm) on the development of EAE in DA rats. They found that stressor exposure prior to EAE induction had no effect, while stressor exposure following EAE induction had a protective effect. Tail-shock reduced the incidence of EAE, delayed the onset of the disease, decreased the severity of clinical and histological symptoms and decreased the duration of the disease. Noise stress on the other hand, delayed the onset of the disease only. In contrast, administration of 60 min of restraint stress on days 2 and 3 post-innoculation with proteolipid protein (PLP, a component of myelin), hastened the onset of EAE symptomatology (Channdler et al., 2002). The acute restraint stress was hypothesized to exacerbate EAE in this instance because it increased

blood brain barrier permeability. However, when restraint stress is repeated, thus chronic instead of acute, it delays the onset of EAE, decreasing the incidence, and reducing clinical symptom severity (Levine et al., 1962; Griffin & Whitacre, 1990; Griffin et al., 1993; Dowdell et al., 1999). Levine and colleagues (1962) only found this effect when restraint was administered during the first few days after EAE induction, but not when starting restraint 9 days following induction - indicating that the stress effects were important in the sensitization stage to the CNS/CFA injection. Subsequently, Levine & Saltzman (1987) also found that restraint stress, or corticosteroid administration, prevented the spontaneous relapse of EAE, when administered during a period of remission. Whitacre and colleagues (Dowdell et al., 1999) found a similar pattern using EAE in B10.PL mice. Chronic restraint stress administered during the initial weeks following EAE induction (and 1 night prior to induction) reduced the incidence, severity, and mortality of the disease, while dramatically delaying the onset. Additionally, the number of relapses was also reduced. This pattern was not observed for shorter durations (1 or 6hrs/day) but was seen when restraint sessions were extensive (12 hrs/day). This indicates that not only the timing of the stressor, but also the severity of the stressor, is important in determining how stress may affect the development of EAE. Blocking of the peripheral effects of glucocorticoids with RU486, reduced the effect of stress on EAE, while antagonizing the β_2-adrenergic receptors had no effect (Dowdell et al., 1999). Adminstration of exogenous glucocorticoids mimicked the effect of restraint stress (Dowdell et al., 1999). Thus, the alleviation of EAE by restraint stress

seems to be mediated by the HPA axis, not the sympathetic nervous system. However, other studies have found that administration of the β adrenergic agonist, isoproterenol, suppressed clinical and histological symptoms of EAE when administered during the first two weeks of infection (Chelmicka Schorr & Arnason, 1999). In a relapsing model of EAE in rats, not only was the severity of the first attack lessened, but the number of relapses was decreased, even if drug administration began after the first attack. In general, repeated stress or administration of stress hormones tends to be immunosuppressive in nature, suppressing the development and exacerbation of EAE.

Stress and TVID. The effects of stress on TVID provide a more complex picture. Whereas in EAE, stress had a generally suppressive effect on disease progression, stress during Theiler's virus infection tends to exacerbate the development of disease (Campbell et al., 2001; Johnson et al., 2001; Satterlee et al., 2001; Sieve et al, 2001). Chronic restraint stress administered 1 night prior to inoculation, and for the following four weeks, profoundly exacerbates TMEV infection during the acute phase (Campbell et al., 2001; Satterlee et al., 2001). During the acute viral infection, mice that are restraint stressed display decreased body weights, increased clinical symptomatology of disease resembling encephalitis, and a dramatically increased mortality rate (80 vs. 5 percent). Thymic atrophy, decreased NK cell activity in the spleen, decreased numbers of lymphocytes in the blood, and decreased inflammation in the CNS all may contribute to the increased viral load found in the CNS of restraint stressed animals (Campbell et al., 2001). Further research has found chemokine

changes in the brain and spleen in response to RST stress (Mi et al., 2004). On day 7 post Theiler's virus infection, Mi et al. (2004) found Ltn, IP-10 and RANTES to be elevated in the spleen and brain, and that RST stress significantly decreased these levels. These chemokines have been shown to be involved in the chemoattraction of inflammatory cells to the CNS (Salmaggi et al., 2002; Palma et al., 2001; Hoffman et al., 1999; Murray et al., 2000; Theil et al., 2000).

Serum corticosterone levels remain high throughout the entire restraint session, and administration of exogenous corticosterone alone mimics the effects of restraint stress: decreased body weights, increased symptomatology, and increased mortality (Satterlee et al., 2001). Presumably, increased levels of corticosterone are causing an overall immunosuppression, which reduces the animal's ability to clear the virus from their CNS. However, the beta-adrenergic antagonist, Nadolol, proved to be somewhat protective against the negative effects of restraint stress on TMEV infection, suggesting a role for not merely the HPA axis, but also the sympathetic branch on the nervous system (McCullough et al., 2002).

Other types of stressors have been shown to exacerbate the acute phase of TMEV, but their effects on the chronic phase have also yet to be determined. Repeated social disruption stress during early infection (Johnson et al., 2001) has been shown to exacerbate acute viral infection, leading to increased clinical scores.

Summary

The role that stress plays in the development and progression of MS, and animal models of MS, is complex. The severity, duration, and timing of the stressor in the disease process can all determine whether stress exacerbates the disease, alleviates the disease, or has no effect at all. In the human literature, chronic stressors such as daily hassels or life changes have been found to precipitate the onset of MS as well as relapses of symptoms. However, the severe stressor or war had the opposite effect. In the animal model EAE, an acute stressor of 60 min of restraint stress applied after disease induction, hastened the onset of EAE symptomatology. However, administration of various chronic or repeated stressors alleviated the symptoms and delayed the onset of EAE in mice and rats, with shock, noise and restraint as stressors. Yet if those stressors were applied prior to the induction, or too long after the induction, they had no effect. The stress effects on EAE appeared to be mediated by the HPA axis, and could be imitated by administration of corticosteroids. Administration of beta-adrenergic agonists also has suppressive effects on EAE. With TVID, restraint stress and social stress applied during the acute viral infection exacerbated the acute phase of the disease, and these stress effects also appeared to be mediated by the HPA axis and sympathetic nervous system. However, the effect of these stressors on the chronic phase of disease remains to be investigated.

Sex, Sex Hormones, and MS

Human MS and an animal model of MS, EAE, have been found to be aleviated during pregnancy (Kim et al., 1999; Runmarker & Anderson, 1995; Confavreux et al., 1998). In MS, the risk of onset was found to be decreased during pregnancy, and returned to normal levels following pregnancy (Runmarker & Anderson, 1995; Confavreux et al., 1998). Pregnancy also decreased the incidence of developing the chronic-progressive form of the disease that commonly follows the initial relapsing-remitting course (Runmarker & Anderson, 1995). Interestingly, at the initial onset of the disease, a greater proportion of women were childless than typically seen in the general population (Runmarker & Anderson, 1995). Hormonal changes during pregnancy may lower the incidence of initially developing, and lower the occurrence and severity of relapses of MS.

Further research using the EAE model in SJL mice, found administration of estrogens (via implantation of estriol pellets) to decrease the severity of the clinical symptoms of the disease. The effective dose of estriol was one that approximated the level of estrogen seen during late pregnancy. Indicative of a TH2 environment, mice treated with estriol were found to have increased T lymphocyte secretion of the TH2 associated cytokine IL-10, as well as higher levels of the autoantibodies for MBP. Progesterone, however, was found to be ineffective in modulating the disease course (Kim et al., 1999).

Though estrogens have been found to alleviate the symptoms of MS, and MS models in animals, other researchers have found a female predominance in

the development of EAE in SJL mice (Voskuhl et al., 1996). This again may be due to the differing effects of various levels of estrogen. Voskuhl and colleagues (1996) used intergender adoptive transfers of T lymphocytes during EAE induction to address this issue of potential sex differences. T cells specific for myelin basic protein (MBP) were isolated from affected females and transferred into males and females. Female recipients had a faster onset of the disease as well as a greater severity of disease symptoms than males. Females had the highest maximal clinical score, and a greater average clinical score. When MBP specific T cells were isolated from males and transferred to both sexes, the same pattern was observed, with female mice being more severely effected. Histopathologic analysis revealed a parallel quantitative difference in the level of disease. Female mice had greater degrees of inflammation and demyelination in the lumbar and thoracic portions of the spinal cord, especially at earlier time points following the transfer of T cells. However, though the disease differed *quantitatively* in clinical and histopathologic severity, no qualitative differences were observed. Sex differences have not only been found in EAE disease progression, but also in the influence of stress on that progression. Male and female Lewis rats were restraint stressed for 9 hrs daily from 5 days before until 18 days after inoculation with myelin basic protein (MBP). Female rats showed a greater suppression of both clinical signs and histological changes than males when stressed.

Sex differences have also been found in a spontaneously occurring animal model of MS: Theiler's murine encephalomyelitis virus (TMEV) induced

demyelination (Hill et al., 1998). In SJL/J mice infected with TMEV, female mice displayed greater inflammation and demyelination of the brain and spinal cord as compared to males. Treatment with Th2 associated, anti-inflammatory cytokines IL-4, IL-10, or an IL-4/IL-10 combination significantly reduced the amount of inflammation and demyelination in the CNS of female mice, but not male mice. Reflecting a Th1 cytokine biased response, anti-TMEV antibodies are of the IgG_{2a} subclass. In the same vein, Th2 antiinflammatory cytokines decreased the levels of virus-specific antibodies in females, but not males (Hill et al., 1998). In contrast to the findings by Hill and colleagues (1998), Alley and colleagues (2003), found male SJL/J to have more severe paralysis and histological signs of inflammation and demyelination in the spinal cord as compared to age-matched infected female SJL/J mice. Male mice also had more severe decrements on two measures of motor ability, the rotarod test and spontaneous activity. Differences in the experimental procedures between the two studies may account for the different results. While both studies used the same strain of mice and strain of Theiler's virus, they differed in the dose of virus (2×10^5 for Hill et al, 1998; 4×10^6 for Alley et al., 2003), the vendor that distributed the mice (male mice from Harlan Sprague Dawley and female mice from Jackson Laboratories for Hill et al., 1998; Jackson Laboratories for Alley et al., 2003), and the housing conditions of the mice. Male mice were housed individually, while female mice were housed in groups (Alley et al., 2003)

An overarching theme throughout the literature is that estrogens decrease symptoms and mortality in Th1 mediated diseases. However, a higher

incidence of these diseases exists in female animals and human populations. The biphasic effects of estrogens could account for this, with low levels leading to a Th1 response, and high levels leading to a Th2 response. Androgens, on the other hand, have proven to be beneficial in suppressing both Th1 and Th2 mediated disease processes.

Conclusions and Current Directions

Though the etiology of autoimmune diseases like MS remains unknown, it has become clear that stress and sex play a role in the onset and progression of the disease. The exact nature of that interaction is complex and is determined by a multitude of factors including the duration, severity, and timing of the stressor in the disease process, as well as the immunological background of the individual, which is in part determined by sexual dimorphism of the immune and stress systems and exposure to sex hormones. In most instances, repeated stress has been found to exacerbate MS, but alleviate the animal model of MS EAE. Similar to MS, repeated stress has been found to exacerbate the other animal model of MS, TVID, during the acute viral infection. What remains to be addressed is the impact that this stress may have on the chronic demyelinating phase of TVID. The current studies investigate the impact of repeated restraint stress on male and female mice of two different strains with genetically different backgrounds. Chapter II addresses the impact of stress on behavioral, immunological, and histological indices of the acute and chronic disease in a strain of mice very susceptible to developing TVID, SJL/J mice. Chapter III

addresses these issues in a strain of mice with intermediate susceptibility to developing TVID, CBA mice. Chapter IV directly compares the impact of stress on the histological lesions of both of these strains using separate stains for inflammation, demyelination, and axonal loss. Chapter V discusses the importance of these findings to the Theiler's Virus, MS, and stress literature.

CHAPTER II

CHRONIC RESTRAINT STRESS DURING EARLY THEILER'S VIRUS INFECTION EXACERBATES THE SUBSEQUENT DEMYELINATING DISEASE IN SJL MICE*

Introduction

To further investigate the role that stress plays in MS, the present study examined whether administration of chronic RST stress during acute infection with Theiler's virus alters the course of the chronic demyelinating disease, Theiler's Virus Induced Demyelination (TVID). Previously we have found that chronic RST stress exacerbates acute TMEV infection in male CBA mice (Campbell et al., 2001). However, susceptibility to Theiler's virus is under genetic control, and also varies according to the sex of mice (Lipton, 1975; Kappel et al., 1990). Whereas CBA mice display an intermediate susceptibility to the development of TVID, SJL mice are highly susceptible to persistent CNS infection with Theiler's virus and the development of TVID. In some studies, female SJL mice are known to have greater susceptibility to disease as compared to males, a pattern that is similar to that found in human MS patients (Kappel et al., 1990; Hill et al., 1998), while under different housing conditions, male mice develop more severe symptomatology of disease (Alley et al., 2003). Thus, disease progression across sex and strain varies, and may differentially interact

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*Reprinted with permission from "Chronic restraint stress during early Theiler's Virus infection exacerbates the subsequent demyelinating disease in SJL mice" by A.N. Sieve, 2004, Journal of Neuroimmunology, in press, 2004 by Elsevier Ltd.

with RST stress. To investigate the effects of chronic RST stress during acute infection on the subsequent TVID, we used male and female SJL mice. SJL mice are the most commonly used strain to study the chronic demyelinating phase of TVID, and the time-course and progression of the demyelinating disease is well characterized (for reviews see: Miller & Gerety, 1990; Tsunoda et al., 1997; Kim & Palna, 1999; Oleszak et al., 2004). Thus, we evaluated the effects of restraint and sex on behavioral, histological, and immunological manifestations of acute and chronic disease.

Materials and Methods

Subjects

Male (n=12) and female (n=12) SJL mice were obtained from Harlan (Houston, TX) at three weeks of age. All mice were housed three per cage with food and water available ad libitum. Male and female mice were housed in separate rooms with separate ventilation systems. They were allowed to acclimate to their environment for one and a half weeks prior to infection, during which time they were handled by all experimenters at least twice and baseline measures were obtained. All animals were housed in accordance with Texas A&M University and National Institutes of Health animal care guidelines.

Infection

The BeAn strain of Theiler's virus (obtained from Dr. H.L. Lipton, Department of Neurology, Northwestern University, Chicago, IL) was propagated and amplified in BHK-21 cells. The culture supernatant containing

infectious virus was aliquoted and stored at -70^{0}C before use (Welsh et al., 1987). As in previous studies, mice were inoculated with 5x10^4 pfu of the BeAn strain of Theiler's virus intracranially into the right cerebral cortex (Welsh et al., 1987; Campbell et al., 2001) at 4.5 weeks of age.

Restraint Stress

Mice were restrained in their home cages, in 60 ml plastic syringes, drilled with holes for ample ventilation (Sheridan et al., 1991; Campbell et al., 2001). RST occurred for a duration of eight h , during the dark cycle, for 5 successive nights per week, with two days off in between weeks. The duration of RST was determined by a pilot study to be the maximum amount of RST stress that uninfected SJL mice of this age could tolerate.

Behavioral Measures

Behavioral Scoring. During the acute phase of Theiler's virus infection, mice were observed and given a numerical score for behavioral indications of encephalitis-like symptoms: 0 = no behavioral signs of illness, 1 = ruffled fur, 2 = ruffled fur and slightly hunched posture, 3 = ruffled fur, very hunched posture, and lethargic, 4 = moribund (Campbell et al., 2001). During the chronic phase of disease, mice were observed and given a numerical score for behavioral signs of the chronic phase: 0 = no behavioral impairment, 1 = weakness in hind limbs, 2 = slightly wobbly gait, 3 = definitely wobbly gait, 4 = very wobbly gait, hunched posture, and loss of righting reflex, 5 = all of the previously mentioned symptoms and incontinence, 6 = moribund (Borrow et al., 1998).

Sucrose Preference. As an additional index of illness, sucrose preference was measured during the acute phase of the disease. Preference for a sweet solution such as sucrose has been shown to decrease following immune challenge with lipopolysaccharide and GP120 administration (Barak et al 2002; Yirmiya et al., 1994, 1996). However, other studies indicate that chronic stress increases preference for sweet food and food intake (Badiani et al., 1996; Ely et al., 1997). Mice were given the option of 2% sucrose solution or tap water for the week prior to infection, and for the following 4 weeks. The position of the sucrose and water bottles was alternated daily, to prevent any place preference. Sucrose preference was calculated by dividing the intake of the sucrose solution, by the total fluid intake.

Rotarod. Mice were placed on a rod (4 cm in diameter, and 20 cm in length) located 20 cm from a padded platform rotating at 6 rpm. Every 30 sec the speed of rotation was increased by 3 rpm (9, 12, 15, 18, 21, 24, 27, 30 rpm). The latency and speed at which the mouse fell from the rod was recorded. Each mouse was run through 2 trials each session. McGavern and colleagues (1999) have found this test to be sensitive to the motor impairments produced by TVID, which are observed with demyelinating lesions in the spinal cord.

Spontaneous Activity. Spontaneous activity has been found to be reduced by immune challenge (lipopolysaccharide, GP120) and Theiler's virus induced demyelinating lesions in the spinal cord (Yirmiya et al., 1994; McGavern, et al., 1999; Barak, et al., 2002). McGavern and colleagues (1999) found that spontaneous activity decreased in Theiler's infected animals as compared to

uninfected controls during the chronic phase of Theiler's virus infection. A modified version of Yirmiya et al (1994) and Ossenkopp et al. (1994) was used to monitor spontaneous activity. To measure activity, mice were placed individually in a 9" (width) x 15" (length) x 24" (height) open field coated with 1/4" of the same bedding used in their home cages. They were videotaped from 24" above the floor, for a 10 min session. The tapes were scored for overall locomotion and frequency of specific behaviors, by experimenters blind to the subjects' conditions. In order to determine locomotion, a grid was placed over the television screen, covering the image of the floor of the open field (five squares across, and three squares down). Passage of the head and shoulders into a new square was considered a square entry. The total number of square entries per min was analyzed. During a separate scoring session, the frequency per min of jumping, leaning, and rearing was recorded to measure vertical activity. Overall horizontal activity was operationalized as the total number of interior and exterior square entries. Overall vertical activity was operationalized as the sum of jumping, rearing and leaning. Separate statistical analyses of the individual subscales of horizontal (interior and exterior grid entries) and vertical (jumping, rearing, and leaning) yielded the same pattern of results as analyzing our horizontal and vertical summary measures.

Assays on Plasma

Blood Collection. Mice were individually transported to an adjacent room and bled via the saphenous vein, within 2 min of cage disturbance to minimize

stress artifacts. The legs were shaved 12 h earlier. The order of blood collection was counterbalanced across conditions. After the bleeding procedure, mice were placed in a recovery cage separate from their homecage, until all of the mice had been bled.

Corticosterone. Plasma cortiocosterone (CORT) was measured by radioimmunoassay (RIA) as described in Keith and colleagues (1978). Following centrifugation and separation, plasma samples were stored at –80 ^{0} C until analyzed. The CORT level in 10 μl of plasma was determined using a ^{125}I-RIA kit (ICN Biomedicals, Inc., Costa Mesa, California).

Antibody Responses to Theiler's Virus and Myelin Proteins. RIAs were used to test mouse plasma for antibodies against Theiler's virus, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein peptide (MOG33-55) and proteolipid protein peptide (PLP139-151) using previously described procedures (Young et al., 1983; Dolimbek et al., 2002). PLP139-151 is the major encephalytic peptide recognized by SJL mice (McRae et al., 1992). This technique was developed because conventional ELISA tests were not sensitive enough to detect these antibodies in the plasma from our mice. The RIA was developed using radio-labeled protein-A which binds to the Fc portion of immunoglobulin. Consequently the level of radioactivity measured equated with the antibody level. As page 56 shows, the antibody levels are fairly low, and by a dilution of 1/160 are not detectable.

Briefly, the plates were washed with Tween 20 (0.05% v/v) in RO H_20 and rinsed with RO H_2O. Washed flexible u-shaped, 96-well polyvinyl chloride

plates (Costar, Cambridge, MA) were coated with 100 μL of carbonate buffer (pH 9.6) containing Theiler's virus (1.0×10^7 p.f.u./100 μL). Likewise, to bind MBP or myelin peptides to the plates, 100 μL assay buffer (made up from two parts: 495 mL of part A: 0.08M Trizma HCl, 0.03M Trizma base and 0.15M NaCl at a final pH of 7.2, and 5 mL of part B: 1.0% non-fat dry milk (NFDM) and 0.5% Tween-20 in reverse osmosis (RO) H_2O containing either 1.0 μg of either MBP (from bovine) (Sigma, USA) MOG33-55 (Sigma, Saint Louis Missouri 63103 USA), or PLP139-151 (AnaSpec Inc., CA) was added to the wells. The plates were incubated at 4^0C for 24 h and then washed and rinsed again as previously described. The plates were blocked with 3.0% NFDM in phosphate PBS (pH 9.0), 200μL/well, for 1 h at 37^0C. Following washing, mouse test serum, negative control mouse serum or positive control serum from mouse (mouse anti-Theiler's virus antisera), or goat polyclonal IgG anti-MBP and goat polyclonal IgG anti-MOG antiserum (Santa Cruz Biotechnology, Inc., CA), respectively were diluted 1/40 in assay buffer, and added to the wells. Positive control Theiler's virus antisera were acquired from pooled serum of 3 SJL mice (Jackson Laboratories) that had received a total of 3 intra-peritoneal (IP) injections of UV-inactivated BeAn (concentration of 1×10^5 p.f.u./100 μL PBS).

Following the serial dilutions, the plates were then incubated for one h at 37^0C and then washed and 100 μl of rabbit anti-mouse IgG (H+L) (diluted 1/500 from stock) (Accurate Chemical & Scientific Corporation, New York) was added to each of the wells in the plates. The plates were incubated for 1 h at 37^0C, washed with Tween 20 (0.05% v/v) in RO H_20 and rinsed with RO H_2O.

Subsequently, 100 µl of ^{125}I-Protein-A (1 x 10^5 cpm/100 µL assay buffer) was added to each well, and the plates were incubated at room temperature for 1 h. They were then washed and rinsed with Tween 20 and RO water (as described above). Once the plates were dry, every well was cut out and counts were determined by using a micromedic 4/200 plus automatic gamma counter.

Histological Analysis

Mice were euthanized at 135 days pi with pentobarbital, perfused via the left ventricle with PBS followed by 10% formalin in phosphate buffer pH 7.2, and processed as described in Campbell et al., (2001). Coronal spinal cord sections were stained with Hematoxylin and Eosin. An experimenter blind to the subjects' conditions scored sections for the severity (number of cell layers in the meninges or perivascular cuffs) and area (percentage on meninges with inflammation and the number of perivascular cuffs) of inflammation. The TMEV model is characterized by inflammatory demyelinating lesions in the spinal cord (Blakemore et al., 1988).

Procedure

A 2 (Sex) X 2 (Stress) design was employed. Six subjects were placed in each group, counter-balanced by weight upon arrival, for a total of 24 subjects. All mice were infected. Half of all mice were RST stressed one night prior to infection, and for the following 4 weeks. Previous studies from our laboratory (Campbell et al., 2001; Welsh et al., 2004) have found that restraint-induced changes in behavioral signs of illness, weight loss, NK cell activity, CNS viral titers, and histological CNS inflammation were selective to infected animals.

Therefore, in the current study only infected animals were used to reduce animal numbers. During acute infection mice were regularly weighed, behaviorally scored for behavioral encephalitis-like symptoms, and had their food, water, and sucrose intake monitored. An additional measure of illness behavior, activity monitoring was taken on D3/4, D10/11, and D17/18 post-infection (pi). Animals were bled via the saphenous vein of the leg on days -5, 1, 7, 16, 24, and 45 pi for CORT analysis. Blood was collected within 2 min of cage disturbance, to minimize any stress artifacts. When RST stressed, animals were bled immediately following the nightly RST session. During the chronic phase of disease, once behavioral signs of the chronic phase began to appear, animals were behaviorally scored for signs of the chronic phase weekly, were tested on the rotarod weekly, and underwent activity monitoring on days 57, 77 and 105 pi. Animals were bled via the saphenous vein of the leg on days 69, 100, and 127 pi for antibody (Ab) to virus and Ab to myelin protein analyses. Mice were euthanized at Day 135 pi with pentobarbital and perfused with PBS followed by 10% formalin.

Statistical Analysis

Analyses of variance (ANOVAs) were conducted on the data. Where possible, baseline measures were used as a covariate, and analyses of covariance

(ANCOVAs) were conducted instead. Bonferroni t-tests, Duncan's multiple range tests, and means comparisons were used for post hoc analyses. Correlation matrices (Pearson's bivariate) were computed on select dependent measures to calculate the inter-relationships between the dependent variables. A p value of 0.05 or less was considered significant in all cases.

Results

Acute Phase

Body Weights. As depicted in Figure 5A, there were significant effects of sex and RST stress on body weights. Independent of condition and day post infection, male mice weighed more than female mice, all Fs $\geq$ 49.60, all ps < 0.05. Over time, RST stressed mice displayed decreased body weights as compared to infected nonrestrained mice, $F(6,119) = 2.768$, $p < 0.05$. This weight difference only existed during the 4 week period of RST stress; upon the cessation of RST stress, there was no significant effect of RST stress on body weights, $F(1,19) = 0.150$, $p > 0.05$.

Food Intake. No significant effect of RST or sex was detected for food intake (data not shown).

Sucrose Preference. There were no baseline differences in sucrose preference. However, during the RST stress period, RST stressed mice had a significantly greater preference for the sucrose solution than nonrestrained

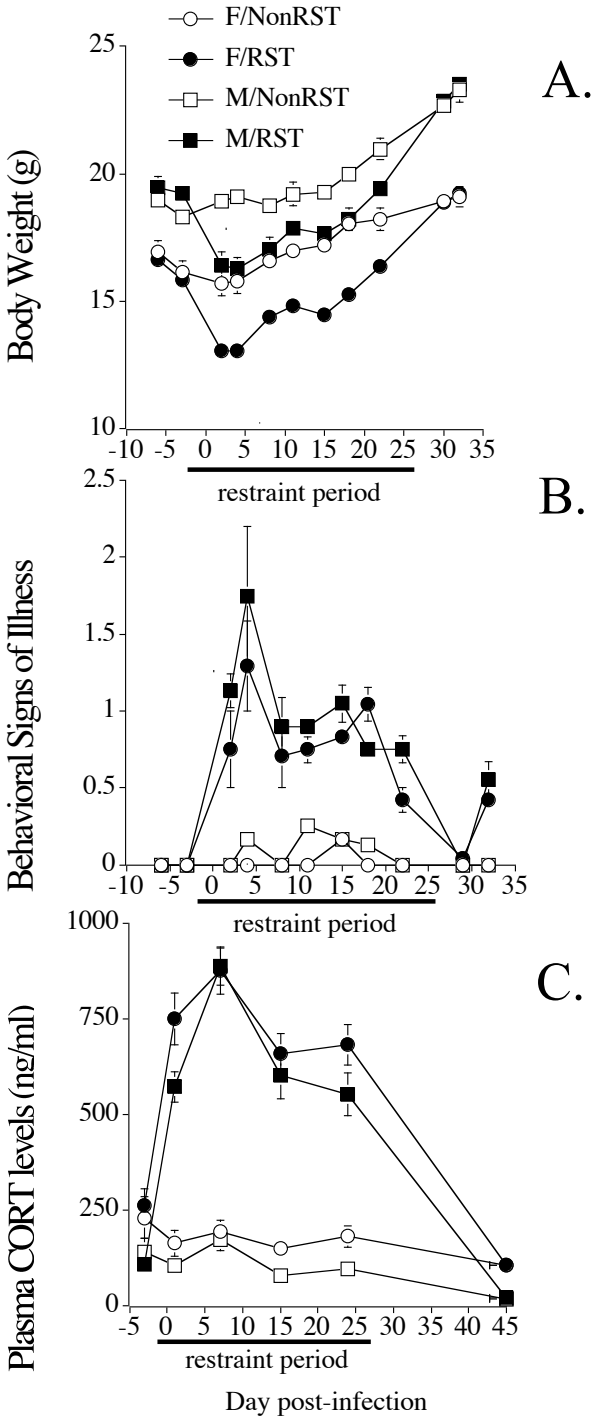

Figure 5. SJL Body Weights, Behavioral Signs of Illness, and Plasma CORT Levels in the Acute Phase. RST decreased body weights (A), increased clinical symptomatology (B) and CORT levels (C) in male and female mice during the restraint stress period. All data are expressed as the mean $\pm$ SEM.

infected mice, $F(1,4) = 8.208$, $p < 0.05$. There were no significant effects of sex orany significant interactions with stress condition. Though a decrease in sucrose preference is associated with increased illness, stress has been found to increase the preference for sweet foods (Badiani et al., 1996; Ely et al., 1997). Here, the effect of stress seems to be overshadowing any potential effect of increased illness on sucrose preference (Table 4).

Table 4. SJL sucrose preference. Sucrose preference is represented as the average daily sucrose solution intake for a cage divided by the total fluid intake for a cage. Sucrose preference was greater for restraint stressed infected animals as compared to nonrestrained infected animals.

		Restraint	**Nonrestraint**
Male	Mean	74.1%	70.9%
	SEM	2.72	0.25
Female	Mean	80.8%	70.2%
	SEM	1.54	0.25

Behavioral Signs of Illness. As depicted in Figure 5B, there was a main effect of RST stress and day pi on behavioral scores, as well as a day pi by stress interaction, all Fs ≥ 3.19, all ps < 0.05. No other differences were found, all ps > 0.05. RST stressed mice displayed increased behavioral scores as compared to infected nonrestrained mice. This behavioral score difference also only persisted during the 4 week period of RST stress. Once again, cessation of RST resulted in

no significant effect of RST stress on behavioral scores, during the acute phase. One mouse died throughout the course of restraint stress (male, restrained). A score of 4 (the highest rating on this scale, which is given for moribund or mortality) was included on the day of the death, and then the deceased animal was removed from all measures for all of the subsequent time points.

Plasma CORT Levels. There was a baseline CORT level difference, such that female mice had higher CORT levels than male mice three days prior to infection, $F(1, 20) = 7.557$, $p < 0.05$. No other baseline differences were found. As depicted in Figure 5C, during the RST stress period, there were main effects of sex, stress, and day pi and a significant stress by day pi interaction, such that over time RST stressed mice had higher CORT levels than infected nonrestrained mice, all Fs ≥ 5.14 , all ps < 0.05. Females continued to have higher CORT levels than males throughout the RST stress period, $F(1,19) = 5.55$, $p < 0.05$. Following the cessation of RST stress, there was no effect of stress condition. Similar to baseline CORT, a significant effect of sex was observed for post-infection CORT with females having higher levels compared to males, $F(1,19) = 33.726$, $p < 0.05$.

Spontaneous Activity. There were significant main effects of stress and day pi on vertical activity (the sum of leaning, rearing and jumping frequencies), both Fs ≥ 6.90, ps < 0.05. Stressed animals had decreased activity as compared to nonrestrained mice. Over time, independent of stress condition, vertical activity also decreased. No other differences were found. On the horizontal activity measure, a similar pattern was observed. There were significant main effects of

stress, day pi, and a stress by day pi interaction, all $\underline{F}s \geq 5.15$, all $\underline{p}s < 0.05$. RST stressed animals had decreased horizontal activity as compared to nonrestrained mice. The nonrestrained mice decreased horizontal activity over time, while horizontal activity levels remained low in the stressed animals. See Figure 6A and 6B for horizontal and vertical activity, respectively.

Summary. The present study replicates the pattern of results previously observed in male CBA mice (Campbell et al., 2001; Satterlee et al., 2001; Faulkner et al., 2003; Welsh et al., 2004) but in a mouse strain with a greater susceptibility to Theiler's virus (SJL mice) as well as in females. However, unlike our prior study (Campbell et al., 2001), we did not see as severe mortality or behavioral signs of illness. In the current study, only one of the 24 SJL mice died (a male, RST, infected). A possible explanation for this difference across studies could be the change in the duration of the nightly RST stress period. To study the SJL mice in the chronic phase, and to reduce mortality due to stress alone (which was observed in SJL mice), the RST stress session was shortened from the 12 h previously used with CBA mice to 8 h in the present study. Even with this change, RST stress still had a significant effect on both male and female SJL mice during the acute phase of Theiler's virus infection, decreasing body weights and activity levels (an indication of increased illness; Barak, 2002), while increasing behavioral signs of illness, plasma CORT levels, and sucrose preference (consistent with previous findings that stress increased the preference for sweet food; Badiani et al., 1996; Ely et al., 1997).

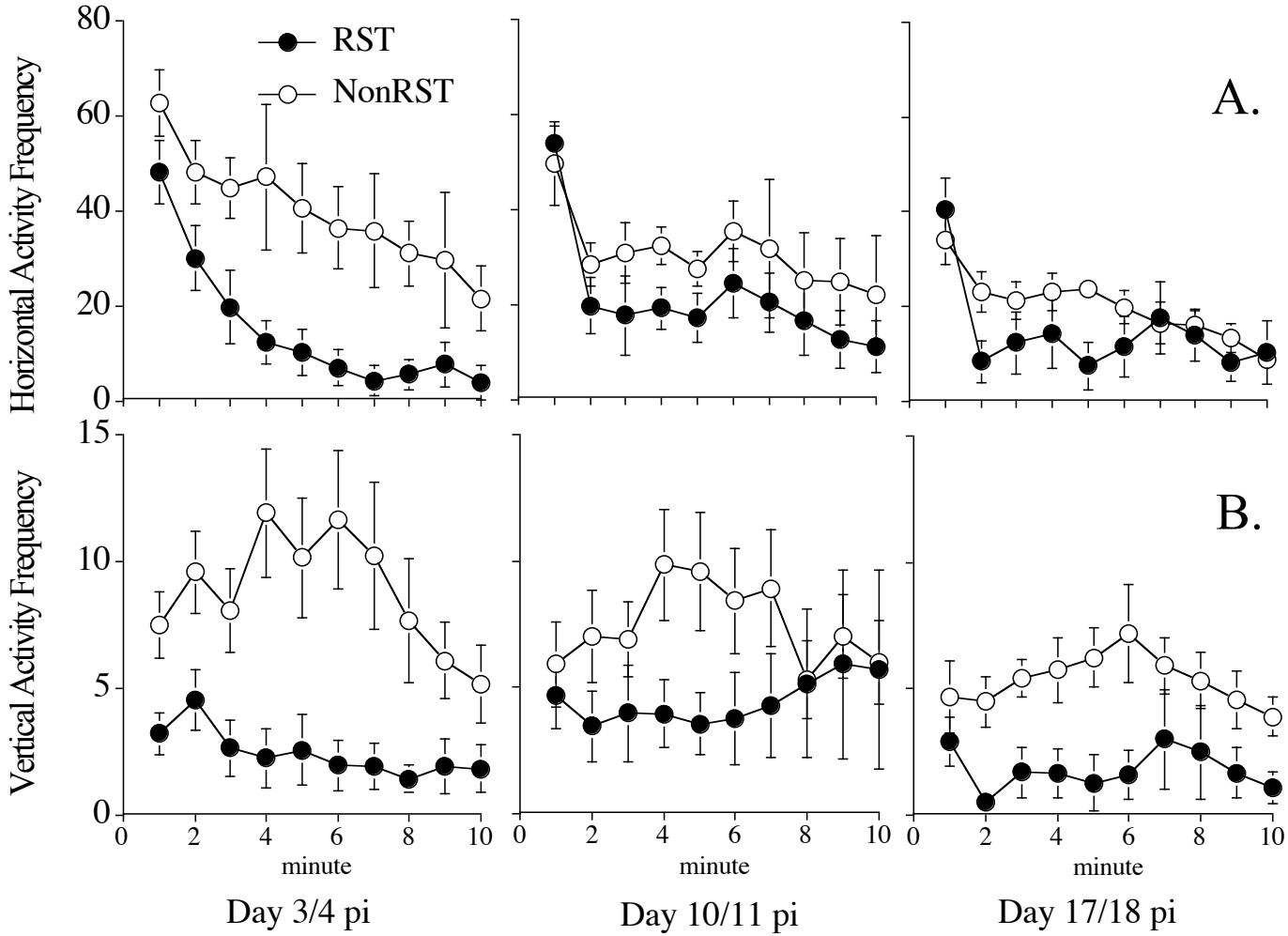

Figure 6 SJL Horizontal and Vertical Activity in the Acute Phase. RST stress decreased horizontal activity (panel A) and vertical activity (panel B) in male and female SJL mice on days 3/4, 10/11, and 17/18 pi. All data are expressed as the mean $\pm$ SEM.

Chronic Phase

Behavioral Data. RST stress exacerbated rotarod performance and behavioral signs during the chronic phase of the disease. However, overall vertical and horizontal activity levels did not appear to be sensitive to the stress-induced exacerbation of disease progression. The shorter duration of our session (10 min versus the 72 h used by McGavern, et al., 1999) may have decreased our ability to detect a significant effect of disease on this measure.

As depicted in Figure 7A, there were significant effects of stress, day pi, and a stress by day pi interaction on behavioral signs of the chronic phase, such that previously RST stressed mice had increased behavioral scores as compared to infected nonrestrained mice, all $\underline{F}s \geq 2.21$, all $\underline{p}s < 0.05$. Bonferroni t-tests confirmed that RST stressed mice had higher behavioral scores at day 45 pi (showing an earlier onset of behavioral symptoms in stressed mice) and 59-134 pi, all $\underline{p}s < 0.05$, but there were no differences between these groups at day 38 and 52 pi, both $\underline{p}s > 0.05$. Although there was a significant main effect of sex, with males consistently having higher behavioral scores than females, $\underline{F}(1,19) = 4.65$, $\underline{p} < 0.05$, there was not a sex by stress interaction.

Similarly, there were significant main effects of sex, stress, and day pi on rotarod performance, such that RST stressed mice spent less time on the rotarod than infected nonrestrained mice, all $\underline{F}s \geq 5.28$, all $\underline{p}s < 0.05$ (Figure 7B). Females had higher latencies to fall from the rotarod, and as time progressed rotarod latencies decreased. There were also significant stress by day pi , and stress by day pi by sex interactions, both $\underline{F}s \geq 2.12$, both $\underline{p}s < 0.05$. Bonferroni t-tests

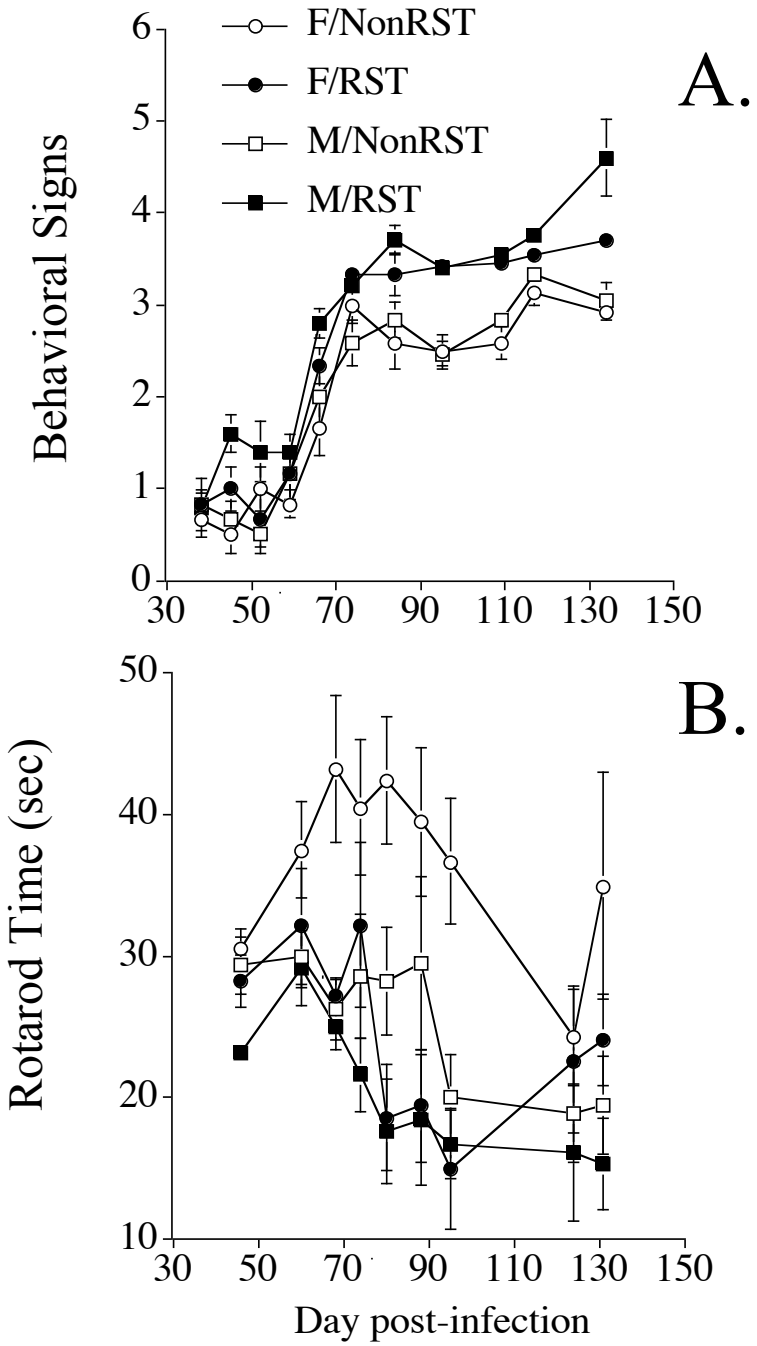

Figure 7. SJL Behavioral Signs and Rotarod in the Chronic Phase. Throughout the chronic phase, previously RST stressed male and female mice had increased behavioral signs of demyelination (panel A). Previously RST stressed male and female mice also had decreased performance on the rotarod task (panel B). All data are expressed as the mean $\pm$ SEM.

confirmed that independent of sex, previously RST stressed mice had significantly worsened rotarod performance at days 46 and 68-95 pi, all $\underline{p}s < 0.05$, but these groups were not different on this measure at days 60, 124, and 131 pi, all $\underline{p}s > 0.05$. A separate set of Bonferroni t-test was used to investigate the stress by day pi by sex interaction. For males, RST stress impaired rotarod performance on days 46, 74, 81, and 88 pi only, all $\underline{p}s < 0.05$. For females, RST impaired rotarod performance on days 68, 81, 88, and 95 pi, but enhanced performance on days 124 and 131 pi as compared to nonrestrained mice, all $\underline{p}s < 0.05$. No other differences were significant.

However, some behavioral measures (spontaneous activity and body weights) were not sensitive to the stress-induced exacerbation of the chronic disease. There were main effects of sex and min, and a day pi by min interaction on horizontal activity, all $\underline{F}s \geq 2.03$, all $\underline{p}s < 0.05$, but no effects of stress. In general, females were more active on this measure, and activity levels were greatest during the first min, and subsequently declined. See Figure 8A and 8B for horizontal and vertical activity levels across time. Consistent with horizontal activity, there were no effects of stress on vertical activity. There was, however, a main effect day pi, $\underline{F}(1,19) = 51.06$, $\underline{p} < 0.05$. Across all groups, vertical activity decreased as the disease progressed. Several factors may contribute to the behavioral deficits observed in infected SJL/J mice during the chronic stage of disease, including persistent virus replication, inflammation, and progressive demyelination that occurs in the CNS of these mice. However, the decrease in

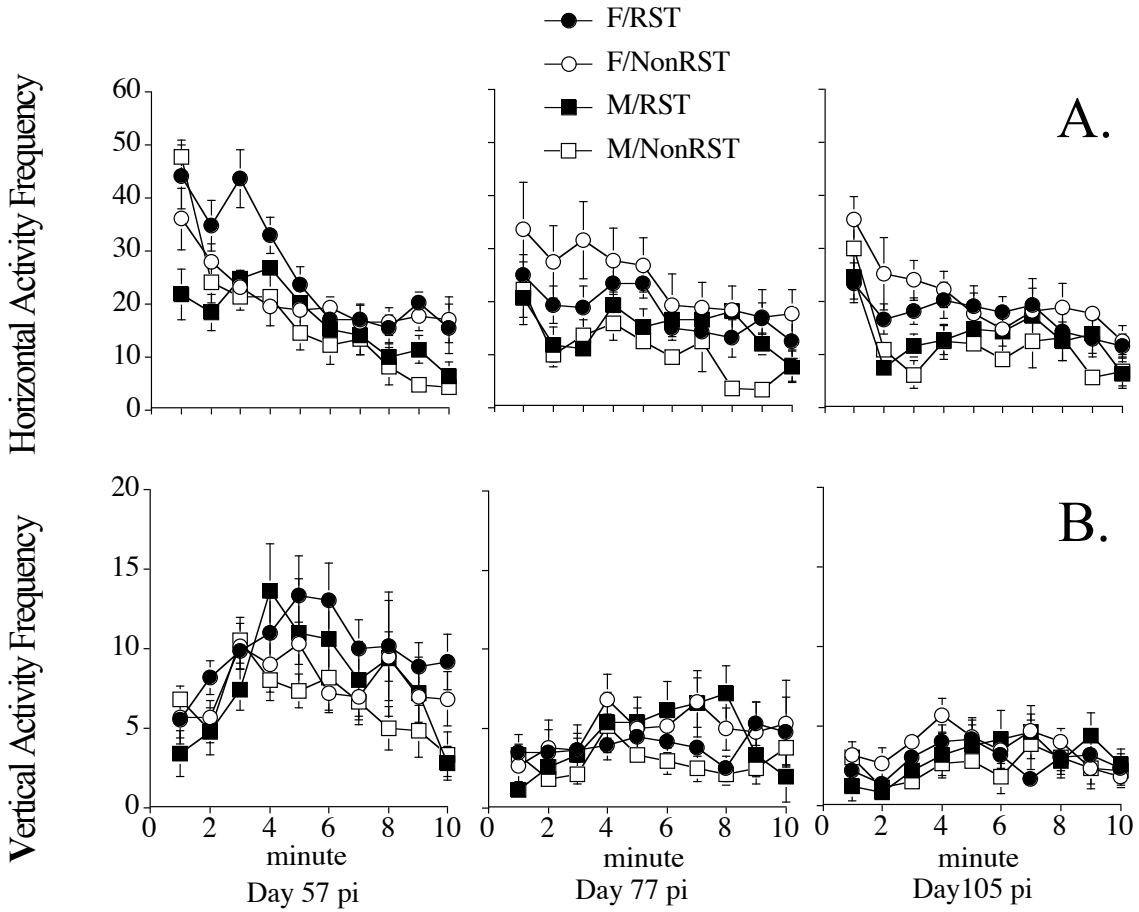

Figure 8. SJL Horizontal and Vertical Activity in the Chronic Phase. Previous RST stress did not alter horizontal activity (panel A) or vertical activity (panel B) in male or female SJL mice on days 57, 77, or 105 pi. However, females were consistently more active than males on both measures. Consistent with previous reports on TVID, vertical activity decreased in all groups as the disease progressed. All data are expressed as the mean $\pm$ SEM.

vertical activity that we observed in all infected mice is consistent with the difference in activity found in other studies between uninfected control mice and infected mice displaying histological signs of demyelination (McGavern et al., 1999). Nevertheless, without an uninfected control group, we cannot definitive conclude that the decrease in vertical activity observed in the present study is attributable to infection.

Body weights also did not show an effect of stress, but merely significant main effects of sex and day pi, such that females consistently weighed less than males, and all mice had normal weight gain with age, both $\underline{F}s \geq 6.81$, both $\underline{p}s < 0.05$ (data not shown).

Taken together, the behavioral data collected during the chronic phase for SJL mice (behavioral signs of the chronic phase and rotarod performance) suggests that RST stress during the acute phase not only exacerbated the acute phase of the disease, but also the later chronic demyelinating condition. It is possible that the lack of sensitivity of activity levels to the stress-induced exacerbation is due to the limited amount of time sampled (10 min). Other researchers have used a time frame as great as 72 h (McGavern, et al., 1999), which was not possible for practical reasons in this experiment. Nonetheless, consistent with McGavern and colleagues (1999), activity levels significantly decreased on both horizontal and vertical measures as the chronic demyelinating phase progressed.

Histological Analysis of Lesions. Analyses of inflammatory lesions within the spinal cord confirmed what was suggested by the behavioral data: in the

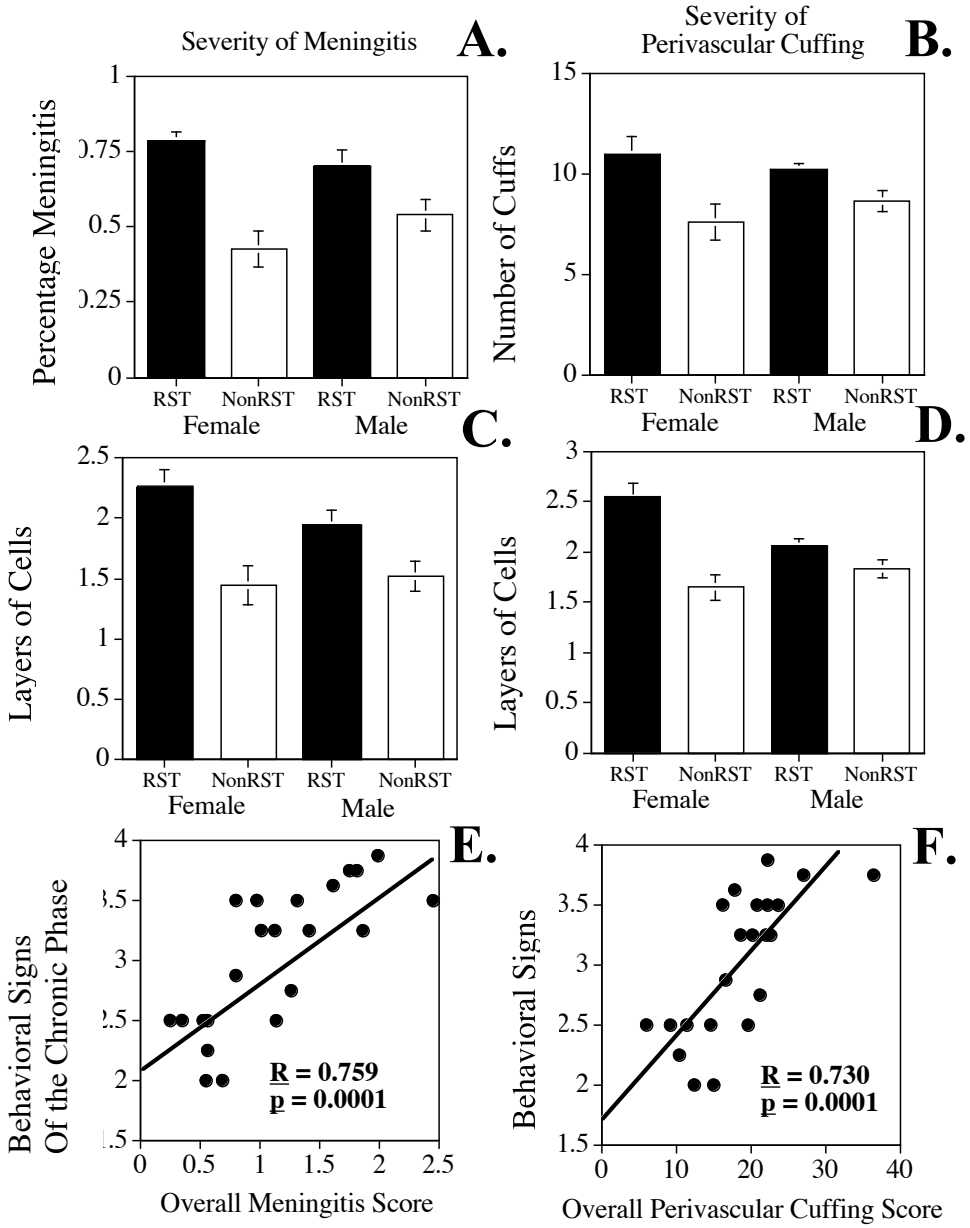

Figure 9. SJL Inflammatory Histological Lesions. Previous RST increased all measures of inflammatory lesions in the spinal cord in male and female SJL mice: percentage of spinal cord with meningitis (panel A) , layers of cells in the meninges (panel C), number of perivascular cuffs in a spinal cord section (panel B), and number of layers of cells in a cuff (panel D). Data are expressed as the mean $\pm$ SEM. Behavioral signs of chronic disease were highly correlated with the severity of meningitis and perivascular cuffing. Overall, perivascular cuffing was computed by multiplying the number of cuffs by the number of cell layers in the cuffs. Overall meningitis was computed by multiplying the percentage of meninges affected by the number of cell layers in the meninges.

chronic phase of the disease, SJL mice, independent of sex, were exacerbated by previous RST stress (Figure 9A-D). A main effect of stress was found on both perivascular cuffing measures (number of cuffs per spinal cord section and average number of cell layers in those cuffs; Figures 9B and 9D respectively), as well as on both measures for meningitis (percentage of meninges inflamed per spinal cord section and the average number of cell layers within the meninges; Figures 9A and 9C respectively), all $\underline{F}s \geq 9.28$, all $\underline{p}s < 0.05$. Previously RST stressed mice had increased inflammatory lesions within the spinal cord as compared to the infected nonrestrained mice. No sex differences were detected. Additionally, both perivascular cuffing and meningitis scores correlated highly with behavioral scores in SJL mice, both $\underline{F}s \geq 22.09$, both $\underline{p}s < 0.05$ (Figure 9E -F).

Plasma Antibody Analyses. Plasma Ab levels to myelin components (MOG33-55, PLP139-151, and MBP) and to Theiler's virus were measured at days 69, 100, and 127 pi. As depicted in Figures 10A, a significant main effect of day pi and a significant day pi by sex interaction were found for Ab to MOG33-55, both $\underline{F}s \geq 3.04$, both $\underline{p}s < 0.05$. Bonferroni t-tests confirmed that at day 69 pi females had significantly more Ab to MOG33-55 than males, $\underline{p} < 0.05$. Previous RST stress did not impact this variable, and no other differences were found, all $\underline{p}s > 0.05$. A significant main effect of sex, and a stress by sex interaction were found for Ab to MBP levels, both $\underline{F}s \geq 5.28$, both $\underline{p}s < 0.05$ (Figure 10B). No other effects were significant. Females had higher levels of Ab to MBP than males, but this main effect was qualified by the sex by stress interaction. Means comparisons determined that only when nonrestrained, females had higher

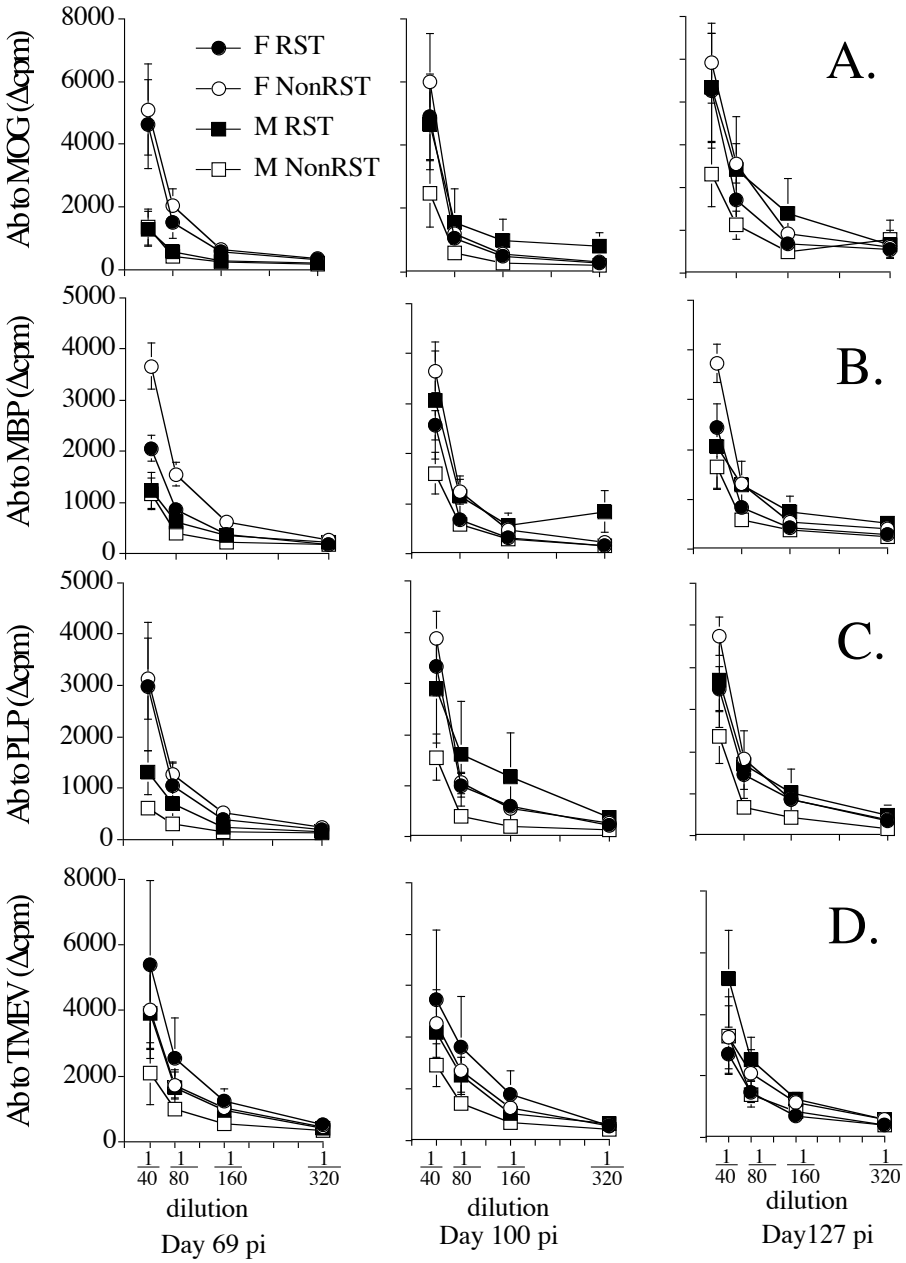

Figure 10. SJL Ab Levels to MOG, MBP, PLP, and TMEV. At day 69 pi, females had greater Ab to MOG33-55 levels than males (panel A). No other sex or stress differences were detected for MOG33-55. Nonrestrained females had higher levels of Ab to MBP than males, and in females only, previous RST stress reduced Ab to MBP levels as compared to nonrestrained mice (panel B). No other sex or stress differences were detected for MBP. No sex or stress differences were detected for PLP139-151 (panel C) or Theiler's virus (panel D). On the right side of the figure, Ab levels from the 1/40 dilution are collapsed across groups and presented over day pi. Ab levels to MOG, PLP, and TMEV increased from Day 69 pi to Day 127 pi. All data are expressed as the mean $\pm$ SEM.

levels than males, $p < 0.05$. Additionally, in females only, previous RST stress reduced Ab to MBP levels as compared to nonrestrained mice, $p < 0.05$. This effect of stress condition did not exist in males. Similarly, as seen in Figure 10C, a significant main effect of day pi was found on Ab to PLP139-151, $F (2, 28) = 6.91$, $p < 0.05$, such that Ab levels to PLP139-151 increased over this time period for all animals independent of sex or stress condition. No other significant effects for PLP139-151 were found. As seen with PLP139-151, a significant main effect of day pi was found on Ab to Theiler's virus, $F (2, 28) = 6.03$, $p < 0.05$, such that Ab levels to Theiler's virus increased over this time period for all animal independent of sex or stress condition (Figure 10D). No other significant differences were found for Ab to Theiler's virus levels, all ps > 0.05. Thus, antibody levels do not reflect the stress-induced exacerbation of disease, suggesting that changes in antibody production do not mediate the adverse effects of restraint. Nevertheless, these findings are important because they characterize the autoantibody response and observed autoantibodies to PLP139-151, MOG33-55 and MBP in the late demyelinating phase of the disease.

Inter-relationships Between Acute and Chronic Phase Dependent Variables

A correlation matrix was computed to investigate the relationship between dependent measures taken during the acute phase of infection with measures taken during the chronic demyelinating phase of disease (Table 5). The acute phase measures of illness and stress were highly correlated with each other. For example, highest CORT level, initial weight loss within the first few days of infection/restraint, highest acute phase behavioral score, as well as

Table 5. Correlations between acute and chronic phase variables. Baseline and acute phase variables are presented by column in the top row, while acute and chronic phase variables are presented in rows by day post-infection. During the acute phase, measures of illness and stress were highly correlated with each other. In addition, acute phase measures of illness and stress were predictive of chronic phase measures of disease progression, including rotarod, behavioral signs of chronic disease and histological inflammatory spinal cord lesions.

Dependent Measure	Day pi	Base-line Weight	Base-line CORT	High Behavioral Score	Initial Wt. Loss	High CORT	Horiz. Activity D3pi	Vertical Activity D3pi
ACUTE PHASE								
Baseline Weight	-3	1.0						
Baseline CORT	-3	**-.747***	1.0					
High Beh. Score	NA	.060	.064	1.0				
Initial Wt. Loss	NA	.029	-.110	**-.807***	1.0			
High CORT	NA	.022	.096	**.687***	**-.863***	1.0		
Horiz. Act.	3	-.074	-.021	**-.839***	**.654***	**-.676***	1.0	
Vert. Act.	3	-.140	.128	**-.686***	**.663***	**-.640***	**.856***	1.0
CHRONIC PHASE								
Behavioral Score	38	-.062	-.074	.106	.002	.105	-.127	-.188
Behavioral Score	45	.319	-.311	**.434***	**-.408***	**.492***	-.329	-.266
Behavioral Score	52	-.005	.023	.079	-.229	.181	.106	.005
Behavioral Score	59	.267	-.250	.070	-.080	.286	.063	.108
Behavioral Score	66	.297	-.179	**.410***	-.269	**.466***	-.360^	-.296
Behavioral Score	74	-.087	.111	.397^	**-.513***	**.479***	-.248	-.214
Behavioral Score	84	.359^	-.140	**.533***	**-.578***	**.559***	-.397^	**-.414***
Behavioral Score	95	.123	-.125	**.745***	**-.794***	**.800***	**-.643***	**-.729***
Behavioral Score	109	.320	-.299	**.683***	**-.661***	**.780***	**-.665***	**-.489***
Behavioral Score	117	.209	-.111	.348^	**-.426***	**.546***	-.327	-.229
Behavioral Score	134	**.446***	-.213	**.559***	**-.573***	**.646***	**-.440***	**-.451***
Rotarod Time	46	-.237	.072	-.366^	.322	**-.499***	.118	.104
Rotarod Time	60	**-.417***	.319	-.094	.215	-.089	-.085	.200
Rotarod Time	68	-.316	.276	-.379^	.282	-.318	.413^	**.461***
Rotarod Time	74	**-.493***	**.461***	-.167	.241	-.179	.091	.262
Rotarod Time	81	-.306	.214	**-.623***	**.549***	**-.549***	**.488***	**.587***
Rotarod Time	88	-.238	.200	**-.495***	**.519***	**-.426***	.285	.393^
Rotarod Time	95	-.240	.182	-.385^	.381^	**-.410***	.368^	**.423***
Rotarod Time	124	-.263	.353^	-.125	.128	-.018	-.027	.206
Ab to MOG33-55	69	-.385^	-.101	-.189	-.093	-.024	.120	-.049
Ab to MOG33-55	100	-.022	-.350	-.038	-.245	.068	.098	-.111
Ab to MOG33-55	127	.029	-.334	-.051	-.234	.095	.027	-.197
Ab to MBP	69	**-.585***	.363	-.324	.154	-.256	**.478***	.142
Ab to MBP	100	-.189	.172	.162	-.140	.042	.105	-.029
Ab to MBP	127	-.398^	.404^	-.029	.028	-.148	.247	.152
Ab to PLP139-151	69	-.360	.044	.117	-.205	.061	-.009	-.153
Ab to PLP139-151	100	-.196	.113	.064	-.226	.083	.042	-.020
Ab to PLP139-151	127	-.028	.058	.045	-.134	.041	.283	.180
Ab to TMEV	69	.081	-.203	.222	-.138	.145	-.123	-.204
Ab to TMEV	100	.102	-.207	.201	-.103	.086	-.069	-.176
Ab to TMEV	127	**.528***	-.354	.143	-.018	.052	-.053	-.091
Number of Cuffs	135	**.417***	-.255	.348^	-.217	.272	-.139	-.137
Layers in Cuffs	135	-.010	.057	**.440***	**.461***	**.493***	-.289	-.261
Percent Meningitis	135	.064	-.101	**.466***	**-.515***	**.652***	-.332	-.346^
Layers in Meninges	135	-.072	.292	**.639***	**-.635***	**.744***	**-.461***	-.380^

* = significant at $p \leq 0.05$ ^= marginally significant at $p \leq 0.10$

horizontal and vertical activity on day 3 pi were all highly correlated, all ps < .05. The same pattern of results was obtained when correlations were conducted for all of the individual time points throughout the acute phase (data not shown). Baseline weight and baseline CORT level did not correlate well with the measures taken during the restraint stress period, but they did correlate highly with each other, such that as mouse weight increased, plasma CORT levels decreased.

Measures of illness and stress during the acute phase were also shown to be predictive of chronic phase disease progression, on rotarod performance, behavioral signs of the chronic phase, and inflammatory lesions in the spinal cord. Highest acute behavioral score, initial weight loss, highest CORT level, and activity measures at day 3 pi were consistently correlated with behavioral signs of the chronic phase and histological lesions, and were frequently correlated with rotarod performance, all ps < .05. However, these acute phase measures were not shown to be predictive of auto-antibody or viral antibody levels in the chronic phase. Baseline weight and CORT levels were also, but to a much lesser degree, predictive of some elements of chronic phase disease. Thus, there appear to be pre-existing individual, or "trait", differences that predicted some aspects of chronic disease, as well as stress-induced, or "state", differences that predicted other manifestations of chronic disease.

Discussion

The present study provides evidence that stress experienced prior to the

onset of a demyelinating condition, such as MS, worsens the development of the disease. RST stress applied during the first 4 weeks of Theiler's virus infection hastened the onset and exacerbated the subsequent demyelinating phase of

Table 6. The effect of restraint on disease progression in SJL mice. **Increase** or **decrease** indicate a significant effect and a dashed line (-----) indicates no effect of restraint stress.

Dependent Measure	Time Period	Male Mice	Female Mice	Sex Difference
ACUTE PHASE				
Body Weights	During Restraint	**decrease**	**decrease**	M>F
	Post Restraint	-----	-----	M>F
Behav. Score	During Restraint	**increase**	**increase**	-----
	Post Restraint	**-----**	**-----**	-----
Plasma CORT	Baseline	NA	NA	F>M
	During Restraint	**increase**	**increase**	F>M
	D45pi	-----	-----	F>M
Food Intake	During Restraint	-----	-----	-----
Sucrose Preference	Baseline	NA	NA	-----
	During Restraint	**increase**	**increase**	-----
Horizontal Activity	During Restraint	**decrease**	**decrease**	-----
Vertical Activity	During Restraint	**decrease**	**decrease**	-----
CHRONIC PHASE				
Body Weights	Chronic Phase	-----	-----	M>F
Rotarod	Chronic Phase	**decrease**	**decrease**	F>M
Behav. Scores	Chronic Phase	**increase**	**increase**	-----
Horizontal Activity	D57,77,& 105pi	-----	-----	F>M
Vertical Activity	D57,77,& 105pi	-----	-----	-----
# PVC	D135pi	**increase**	**increase**	-----
Layers in PVCs	D135pi	**increase**	**increase**	-----
% Meningitis	D135pi	**increase**	**increase**	-----
Layers in Meninges	D135pi	**increase**	**increase**	-----
Ab to MOG33-55	D69pi	-----	-----	F>M
Ab to MBP	D69, 100, & 127pi	-----	**decrease**	FC>MC
Ab to PLP139-151	D69, 100, & 127pi	-----	-----	-----
Ab to TMEV	D69, 100, & 127pi	------	-----	-----

disease. See table 6 for a summary of the findings. Both male and female SJL mice that were previously stressed displayed increased behavioral and histological indications of disease: exacerbated behavioral signs of chronic disease, decreased rotarod performance, and increased inflammatory lesions within the spinal cord. Moreover, acute phase measures of illness and stress predicted chronic phase measures of disease progression, including rotarod, behavioral indications of chronic disease, and inflammatory lesions in the spinal cord.

These findings provide experimental evidence that stress administered during early infection can exacerbate the subsequent inflammatory demyelinating phase of disease. Though this does not coincide with studies using EAE, which show no effect of stress prior to disease induction, and a suppression during disease induction (Levine et al., 1962; Griffin & Whitacre, 1990; Griffin et al., 1993; Dowdell, 1999; Levine et al., 1987; Bukilica et al., 1991), it is similar to that reported by MS patients (Mohr & Cox, 2001). Thus, this study provides some of the first experimental evidence in an animal model that corresponds to the correlational evidence in human MS populations. Mohr and Cox (2001) reviewed the effects of stress and MS finding that stressors such as daily hassels and life changes precipitated the onset and exacerbations of MS in retrospective accounts and longitudinal studies. A subsequent meta-analysis by Mohr and colleagues (2004) found the effect size for stress exacerbating MS to be greater than that of the reduction in symptoms from the principle drugs used to treat MS. Though Nisipeanu and Korcyzn (1993) found the severe stress of war

to reduce relapse, Ackerman and colleagues (2003) found stress to exacerbate MS independent of the type of stressor.

The current study also replicates, in male and female SJL mice, the previous findings in male CBA mice: RST stress exacerbates early Theiler's virus infection (Campbell et al., 2001). Both male and female SJL mice displayed decreased body weights, and increased behavioral signs of illness during the first 4 weeks of infection. However, mortality rates were lower in the current study when compared to Campbell et al. (2001). This may be related to the reduced amount of RST stress administered in the current study (8 h instead of 12 h per night). The duration of restraint was reduced from 12 to 8 h to reduce mortality due to stress alone, which was observed in SJL mice, but not in CBA mice. In addition, the present study extends this line of work by showing that RST stress exacerbated another index of illness behavior during acute disease: activity monitoring. RST stressed animals displayed decreased activity levels as compared to infected non-restrained mice, providing converging behavioral evidence that early viral infection is exacerbated by stress. Furthermore, this increase in illness was associated with an increase in CORT levels throughout the four weeks of RST stress.

Another aim of this study was to investigate whether stress-induced changes in disease course varied by sex. We expected that CORT levels in all RST stressed mice would be elevated, possibly to a greater degree in females. Female rodents are known to have a greater, and more long-lasting HPA activation in response to stress (Turner, 1990; Homo-Delarche et al., 1991; Griffin

et al., 1993; Gaillard & Spinedi, 1998), and this could have modulated the effects of stress on Theiler's virus infection. Indeed, females not only displayed a higher basal CORT level, but also greater stress-induced levels of CORT. However, this sex difference in CORT levels did not translate into sex by stress interaction on Theiler's virus infection. While stress exacerbated infection, it did not do so to a greater degree in females even with their significantly higher CORT levels.

Yet, sex did impact the disease process, *independent* of stress condition. Though sex differences were found, the pattern of results is complex. In the early viral infection, there were no sex differences in behavioral signs of illness, activity monitoring, or sucrose preference. However, in later disease, males had greater behavioral signs of chronic disease, poorer rotarod performance, and reduced activity levels as compared to females, but there were no sex differences in spinal cord lesions. While males had greater behavioral signs of disease, females had higher levels of autoantibody levels to MOG33-55 and MBP at certain time points in the late disease. The pattern of results across other studies is similarly complicated. While Alley et al. (2003) found SJL males to develop a greater severity of disease than females, Hill et al. (1998) found female SJL mice to have a greater incidence and severity of disease. In SJL mice infected with TMEV, female mice displayed greater inflammation and demyelination of the brain and spinal cord as compared to males (Hill et al., 1998).

Multiple factors may underlie the divergent findings on sex differences in the development of TVID, including strain of Theiler's virus, and environmental

variations. Alley et al. (2003) and Hill et al. (1998) used the DA strain of Theiler's virus while the current study used the BeAn strain. Another possible explanation may lie in variations in housing conditions. All of the mice in the current study were housed in groups of 3; whereas in Alley et al. (2003) males were individually housed while females were group housed. Because social isolation is a significant stressor (Banerjee, 1972), the sex differences observed in the Alley study may be attributable to this variable. Thus, the effect of sex in Theiler's virus infection requires further investigation.

The findings that stress exacerbates the early viral infection (Campbell et al., 2001; current study) and the later demyelinating disease (current study) in Theiler's virus infection contrast with the effects of stress on the other commonly used rodent model of multiple sclerosis, EAE. The differences in how stress effects EAE and Theiler's virus infection may lie in their immunological mechanisms of demyelination and neuronal destruction. The mechanism of EAE pathogenesis involves the induction of autoreactive T cells. Stimulated and activated CD4+ T cells increase adhesion molecules, enter the CNS, and secrete proinflammatory Th1 cytokines, leading to the recruitment of mononuclear cells. B cell secretion of anti-myelin antibody (Ab), in concert with macrophage/glia secreted cytotoxic factors, leads to demyelination (Tsunoda and Fujinami, 1996). In this exclusively autoimmune mediated model, stress induced suppression of the immune system, and thus alleviation of the disease process, would be an expected result. Indeed, when stress does alter the course of EAE it tends to reduce various aspects of the disease. For example, prior

studies indicate that repeated electric shock or noise stress have no effect when administered prior to EAE induction, while stressor exposure following EAE induction had a protective effect (Bukilica et al., 1991). Other studies found restraint stress in rats to likewise delay EAE onset and decrease the incidence and severity of disease (Levine et al., 1962; Griffin & Whitacre, 1990; Griffin et al., 1993; Dowdell et al. 1999). These and other studies have also shown that the alleviation of EAE by restraint appears to be mediated by the HPA axis, rather than the sympathetic nervous system (Dowdell et al., 1999; Levine, 1987).

Theiler's virus induced demyelination (TVID), though similar to EAE in the resulting demyelination, behavioral signs of chronic disease, and histological lesions, involves a different immunopathological pathway (for reviews see: Dal Canto et al., 1995; Tsunoda and Fujimani, 1996; Oleszak et al., 2004). Following the acute viral infection, where Theiler's virus specific immunity induces apoptosis in virally infected neurons, the persistent viral infection in oligodendrocytes and macrophages is attacked by Theiler's virus specific and myelin reactive, cytotoxic, T-helper, and antibody responses. Multiple mechanisms of demyelination have been found in TVID, including: direct viral lysis of oligodendrocytes (Roos and Wollmann, 1984), bystander demyelination mediated by virus specific DTH T cells (Clatch et al., 1987), cytotoxic T cell reactivity (Rodriguez and Sriram, 1988), and autoimmune mediated demyelination (Welsh et al., 1987; Miller et al., 1997; Borrow et al., 1998). In the current study, antibody levels to virus and myelin proteins (MOG33-55, PLP139-151, MBP) were measured at days 69, 100, and 127 pi. Autoantibodies to whole

myelin membranes (Welsh et al., 1987) and MBP (Rauch et al., 1987) have been previously detected in mice infected with Theiler's virus. In the current study we further dissected the autoantibody response and observed autoantibodies to PLP139-151, MOG33-55, and MBP in the late demyelinating phase of the disease. To the best of our knowledge this represents the first report of specific autoantibodies directed against these determinants in TVID and as such, validates Theiler's virus infection as an excellent model of MS. It is interesting to note that T cell responses to these proteins also develop during late TVID (Miller et al., 1997).

When comparing the effects of stress on EAE and TVID, it is important to note that the stressors are being applied during different phases in the immunological response of the disease process. In EAE, a purely autoimmune mediated model, the stressors are applied either before or during the autoimmune components of the disease. With research conducted on Theiler's virus infection thus far, the stressor has been applied before and during the acute viral infection, when the immune system is attempting to remove the virus from the CNS (Campbell et al., 2001; Welsh et al., 2004; Mi et al., 2004). It is possible that if the stress-induced suppression of the immune system occurred during the chronic demyelinating disease, when autoimmune processes are in place, rather that the preceding acute viral infection, that a similar pattern to EAE would be discovered (Lipton & DalCanto, 1976; 1977). Stress at that point in the disease process may alleviate rather than exacerbate the TVID. Research is currently underway to address this issue.

There are at least two potential pathways by which stress during early infection could exacerbate Theiler's virus induced demyelination. One possibility is that stress alters the functioning of the immune system. Biron (1998, 1999) proposes that altering the early immunological events in a viral infection may have a cascading effect on later infection. The initial responses to the virus influence the innate immune response and the production of innate components, which in turn influence the development of the adaptive immunity directed towards the viral infection. Early cytokine responses can activate protective mechanisms within cells as well as natural killer cells (NK cells) and macrophages. This innate cytokine and cellular response can shape the endogenous T cell responses. Thus, altering the initial cytokine and innate immune responses to Theiler's virus with RST stress, may subsequently alter the adaptive immune responses to the virus and myelin in the chronic phase of disease.

Consistent with this view, we have found that male CBA mice subjected to RST during acute infection exhibit thymic atrophy, decreased NK cell activity in the spleen, and decreased numbers of lymphocytes in the blood, all of which may contribute to the increased viral load found in the CNS of RST stressed animals (Campbell et al., 2001; Welsh et al., 2004). Further research has found chemokine changes in the brain and spleen in response to RST stress (Mi et al., 2004). On day 7 post Theiler's virus infection, Mi et al. (2004) found Ltn, IP-10 and RANTES to be elevated in the spleen and brain, and that RST stress significantly decreased these levels. These chemokines have been shown to be

involved in the chemoattraction of inflammatory cells to the CNS (Salmaggi et al., 2002; Palma et al., 2001; Hoffman et al., 1999; Murray et al., 2000; Theil et al., 2000). Indeed, stress during early Theiler's virus infection has been found to decrease inflammation in the CNS at D7p.i. (Mi et al., 2004; Campbell et al., 2001), followed by an increase in inflammation at D24p.i. (Campbell et al., 2001). Would there be similar cellular changes in the chronic demyelinating phase of Theiler's virus infection when RST stressed in the early viral infection? Additionally, chronic restraint stress may not only result in persistent immunological changes that last throughout the chronic phase of disease, but these immunological changes may be due to a chronic stress-induced persistent alteration in neuroendocrine function, including the activity of the hypothalamic pituitary adrenal axis (McEwen, 1998). Both of these possibilities require further investigation. However, data from this study suggest that humoral immunity may be unchanged in the chronic phase when mice are RST stressed during early viral infection. We did detect specific autoantibodies directed against MOG33-55, PLP139-151, and MBP in TVID, further validating Theiler's virus infection as an excellent model of MS. However, overall antibody levels did not reflect the stress-induced exacerbation of disease. This may reflect insufficient sensitivity on this measure, or be an indication that stress-induced changes in the disease process do not include antibody production.

A second possibility lies within the effects of stress on the viral load within the CNS. RST stress has been previously found to decrease Theiler's virus clearance from the brain and spinal cord during the first 4 weeks of

infection (Campbell et al., 2001). This suppression of viral clearance is thought to be at least in part due to a stress-induced corticosterone mediated immunosuppression. Previous studies (Satterlee et al., 2001) have found that administration of corticosterone as a substitute for restraint stress lead to a similar worsening of the acute viral infection. Following acute viral infection, Theiler's virus levels normally drop to undetectable levels until the onset of the chronic demyelinating phase of disease, where a resurgence of viral replication can be seen (Welsh et al., 1987; Welsh et al., 1989). Did the stress-induced increase in viral load in the acute phase translate into a higher viral load at the onset of the chronic phase? Did this increased level of viral replication lead to increased myelin destruction and enhanced autoimmune demyelination? Borrow et al. (1992) found that removing CD8 T cells prior to acute viral infection resulted in a higher viral load in the CNS and more severe demyelinating disease. Stress-induced immunosuppression may likewise be exacerbating the demyelinating disease through this mechanism.

Future studies intend to investigate the potential mechanisms underlying the stress-induced exacerbation of the chronic demyelinating phase of Theiler's virus infection. Though many avenues of research still exist in this area, the present study provides strong experimental evidence, that parallels correlational evidence in human Multiple Sclerosis patients, that stress worsens the development of a demyelinating condition.

CHAPTER III
SEX DEPENDENT EFFECTS OF CHRONIC RESTRAINT STRESS DURING EARLY THEILER'S VIRUS INFECTION ON THE SUBSEQUENT DEMYELINATING DISEASE IN CBA MICE

Introduction

The effect of chronic restraint stress during the acute phase of Theiler's virus in male and female SJL mice replicated the previous finding in the acute phase with male CBA mice. Though the same pattern of results was found in male CBA and SJL mice during the acute viral infection, there is reason to believe that the effect of stress on the chronic demyelinating phase may vary across strain. When nonstressed, CBA and SJL mice similarly display asymptomatic acute viral infections (Campbell et al., 2001; Chapter II). However, while typically 100% of SJL mice have viral persistence in the CNS and develop the chronic demyelinating phase of the disease, CBA mice have only an intermediate susceptibility to the chronic phase of the disease (for reviews see: Friedmann & Lorch, 1985; Oleszak et al., 2004). Simas and Fazakerley (1996) found that when infected with 10^4 p.f.u. of the BeAn strain of Theiler's virus, the resulting disease course of CBA mice could be separated into three categories: 1) death by acute encephalitis, 2) no clinical signs in the acute phase, but high viral titers in the acute phase that led to persistence of virus in the CNS throughout the chronic phase of the disease, 3) no clinical signs in the acute phase, low viral titers in the acute phase, and no detectable virus after 28 days post-infection. Thus, while the outward symptoms of the acute viral

infection in CBA and SJL mice may appear the same, underlying differences in viral persistence in the CNS may still exist.

To further investigate the interaction between sex and stress in the development of Theiler's Virus Induced Demyelination the present study examined whether administration of chronic RST stress during acute infection with Theiler's virus alters the course of the chronic demyelinating disease in male and female CBA mice. Thus, we evaluated the effects of restraint and sex on behavioral, histological, and immunological manifestations of acute and chronic disease.

Materials and Methods

Subjects

Male (n=12) and female (n=12) CBA mice were obtained from Harlan (Houston, TX) at three weeks of age. All mice were housed three per cage with food and water available ad libitum. Male and female mice were housed in separate rooms with separate ventilation systems. They were allowed to acclimate to their environment for one and a half weeks prior to infection, during which time they were handled by all experimenters at least twice and baseline measures were obtained. All animals were housed in accordance with Texas A&M University and National Institutes of Health animal care guidelines.

Infection

The BeAn strain of Theiler's virus (obtained from Dr. H.L. Lipton, Department of Neurology, Northwestern University, Chicago, IL) was propagated and amplified in BHK-21 cells. The culture supernatant containing infectious virus was aliquoted and stored at -70^{0}C before use (Welsh et al., 1987). As in previous studies, mice were inoculated with 5x10^4 pfu of the BeAn strain of Theiler's virus intracranially into the right cerebral cortex (Welsh et al., 1987; Campbell et al., 2001) at 4.5 weeks of age.

Restraint Stress

Mice were restrained in their home cages, in 60 ml plastic syringes, drilled with holes for ample ventilation (Sheridan et al., 1991; Campbell et al., 2001). RST occurred for a duration of twelve h, during the dark cycle, for 5 successive nights per week, with two days off in between weeks.

Behavioral Measures

Behavioral Scoring. During the acute phase of Theiler's virus infection, mice were observed and given a numerical score for behavioral indications of encephalitis-like symptoms: 0 = no behavioral signs of illness, 1 = ruffled fur, 2 = ruffled fur and slightly hunched posture, 3 = ruffled fur, very hunched posture, and lethargic, 4 = moribund (Campbell et al., 2001). Likewise, during the chronic phase of disease, mice were again observed and given a numerical score for behavioral signs of the chronic phase: 0 = no behavioral impairment, 1 = weakness in hind limbs, 2 = slightly wobbly gait, 3 = definitely wobbly gait, 4 = very wobbly gait, hunched posture, and loss of righting reflex, 5 = all of the

previously mentioned symptoms and incontinence, 6 = moribund (Borrow et al., 1998).

Sucrose Preference. As an additional index of illness, sucrose preference was measured during the acute phase of the disease. Preference for a sweet solution such as sucrose has been shown to decrease following immune challenge with lipopolysaccharide and GP120 administration (Barak et al 2002; Yirmiya et al., 1994, 1996). However, other studies indicate that chronic stress increases preference for sweet food and food intake (Badiani et al., 1996; Ely et al., 1997). Mice were given the option of 2% sucrose solution or tap water 14-31 days pi. The position of the sucrose and water bottles was alternated daily, to prevent any place preference. Sucrose preference was calculated by dividing the intake of the sucrose solution, by the total fluid intake.

Rotarod. Mice were placed on a rod (4 cm in diameter, and 20 cm in length) located 20 cm from a padded platform rotating at 6 rpm. Every 30 sec the speed of rotation was increased by 3 rpm (9, 12, 15, 18, 21, 24, 27, 30 rpm). The latency and speed at which the mouse fell from the rod was recorded. Each mouse was run through 2 trials each session. McGavern and colleagues (1999) have found this test to be sensitive to the motor impairments produced by TVID, which are observed with demyelinating lesions in the spinal cord.

Spontaneous Activity. Spontaneous activity has been found to be reduced by immune challenge (lipopolysaccharide, GP120) and Theiler's virus induced demyelinating lesions in the spinal cord (Yirmiya et al., 1994; McGavern, et al., 1999; Barak, et al., 2002). McGavern and colleagues (1999) found that

spontaneous activity decreased in Theiler's infected animals as compared to uninfected controls during the chronic phase of Theiler's virus infection. A modified version of Yirmiya et al (1994) and Ossenkopp et al. (1994) was used to monitor spontaneous activity. To measure activity, mice were placed individually in a 9" (width) x 15" (length) x 24" (height) open field coated with 1/4" of the same bedding used in their home cages. They were videotaped from 24" above the floor, for a 10 min session. The tapes were scored for overall locomotion and frequency of specific behaviors, by experimenters blind to the subjects' conditions. In order to determine locomotion, a grid was placed over the television screen, covering the image of the floor of the open field (five squares across, and three squares down). Passage of the head and shoulders into a new square was considered a square entry. The total number of square entries per min was analyzed. During a separate scoring session, the frequency per min of jumping, leaning, and rearing was recorded to measure vertical activity. Overall horizontal activity was operationalized as the total number of interior and exterior square entries. Overall vertical activity was operationalized as the sum of jumping, rearing and leaning. Separate statistical analyses of the individual subscales of horizontal (interior and exterior grid entries) and vertical (jumping, rearing, and leaning) yielded the same pattern of results as analyzing our horizontal and vertical summary measures.

Assays on Plasma

Blood Collection. Mice were individually transported to an adjacent room and bled via the saphenous vein, within 2 min of cage disturbance to minimize stress artifacts. The legs were shaved 12 h earlier. The order of blood collection was counterbalanced across conditions. After the bleeding procedure, mice were placed in a recovery cage separate from their homecage, until all of the mice had been bled.

Corticosterone. Plasma cortiocosterone (CORT) was measured by radioimmunoassay (RIA) as described in Keith and colleagues (1978). Following centrifugation and separation, plasma samples were stored at –80 ^{0} C until analyzed. The CORT level in 10 μl of plasma was determined using a ^{125}I-RIA kit (ICN Biomedicals, Inc., Costa Mesa, California).

Antibody Responses to Theiler's Virus and Myelin Proteins. RIAs were used to test mouse plasma for antibodies against Theiler's virus, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein peptide (MOG33-55) and proteolipid protein peptide (PLP139-151) using previously described procedures (Young et al., 1983; Dolimbek et al., 2002). PLP139-151 is the major encephalytic peptide recognized by SJL mice (McRae et al., 1992). This technique was developed because conventional ELISA tests were not sensitive enough to detect these antibodies in the plasma from our mice. The RIA was developed using radio-labeled protein-A which binds to the Fc portion of immunoglobulin. Consequently the level of radioactivity measured equated with the antibody

level. As Figure 6 shows, the antibody levels are fairly low, and by a dilution of 1/160 are not detectable.

Briefly, the plates were washed with Tween 20 (0.05% v/v) in RO H_20 and rinsed with RO H_2O. Washed flexible u-shaped, 96-well polyvinyl chloride plates (Costar, Cambridge, MA) were coated with 100 μL of carbonate buffer (pH 9.6) containing Theiler's virus (1.0×10^7 p.f.u./100 μL). Likewise, to bind MBP or myelin peptides to the plates, 100 μL assay buffer (made up from two parts: 495 mL of part A: 0.08M Trizma HCl, 0.03M Trizma base and 0.15M NaCl at a final pH of 7.2, and 5 mL of part B: 1.0% non-fat dry milk (NFDM) and 0.5% Tween-20 in reverse osmosis (RO) H_2O containing either 1.0 μg of either MBP (from bovine) (Sigma, USA) MOG33-55 (Sigma, Saint Louis Missouri 63103 USA), or PLP139-151 (AnaSpec Inc., CA) was added to the wells. The plates were incubated at 4^0C for 24 h and then washed and rinsed again as previously described. The plates were blocked with 3.0% NFDM in phosphate PBS (pH 9.0), 200μL/well, for 1 h at 37^0C. Following washing, mouse test serum, negative control mouse serum or positive control serum from mouse (mouse anti-Theiler's virus antisera), or goat polyclonal IgG anti-MBP and goat polyclonal IgG anti-MOG antiserum (Santa Cruz Biotechnology, Inc., CA), respectively were diluted 1/40 in assay buffer, and added to the wells. Positive control Theiler's virus antisera were acquired from pooled serum of 3 SJL mice (Jackson Laboratories) that had received a total of 3 intra-peritoneal (IP) injections of UV-inactivated BeAn (concentration of 1×10^5 p.f.u./100 μL PBS).

Following the serial dilutions, the plates were then incubated for one h at 37^0C and then washed and 100 µl of rabbit anti-mouse IgG (H+L) (diluted 1/500 from stock) (Accurate Chemical & Scientific Corporation, New York) was added to each of the wells in the plates. The plates were incubated for 1 h at 37^0C, washed with Tween 20 (0.05% v/v) in RO H_20 and rinsed with RO H_2O. Subsequently, 100 µl of ^{125}I-Protein-A (1×10^5 cpm/100 µL assay buffer) was added to each well, and the plates were incubated at room temperature for 1 h. They were then washed and rinsed with Tween 20 and RO water (as described above). Once the plates were dry, every well was cut out and counts were determined by using a micromedic 4/200 plus automatic gamma counter.

Histological Analysis

Mice were euthanized at 277 days pi with pentobarbital, perfused via the left ventricle with PBS followed by 10% formalin in phosphate buffer pH 7.2, and processed as described in Campbell et al., (2001). Coronal spinal cord sections were stained with Hematoxylin and Eosin. An experimenter blind to the subjects' conditions scored sections for the severity (number of cell layers in the meninges or perivascular cuffs) and area (percentage on meninges with inflammation and the number of perivascular cuffs) of inflammation. The TMEV model is characterized by inflammatory demyelinating lesions in the spinal cord (Blakemore et al., 1988). From our analysis of serial sections of spinal cord stained with Wiles myelin stain and hematoxylin and eosin (to visualize inflammation) we have found that demyelination is observed in the areas of inflammation (Sieve et al., in preparation).

Procedure

A 2 (Sex) X 2 (Stress) design was employed. Six subjects were placed in each group, counter-balanced by weight upon arrival, for a total of 24 subjects. All mice were infected. Half of all mice were RST stressed one night prior to infection, and for the following 4 weeks. Previous studies from our laboratory (Campbell et al., 2001; Welsh et al., 2004) have found that restraint-induced changes in behavioral signs of illness, weight loss, NK cell activity, CNS viral titers, and histological CNS inflammation were selective to infected animals. Therefore, in the current study only infected animals were used to reduce animal numbers. During acute infection mice were regularly weighed, behaviorally scored for behavioral encephalitis-like symptoms, and had their food, water, and sucrose intake monitored. An additional measure of illness behavior, activity monitoring was taken on D10/11 and D17/18 post-infection (pi). Animals were bled via the saphenous vein of the leg on days -5, 1, 7, 16, 24, and 35 pi for CORT analysis. Blood was collected within 2 min of cage disturbance, to minimize any stress artifacts. When RST stressed, animals were bled immediately following the nightly RST session. During the chronic phase of disease, once behavioral signs of the chronic phase began to appear, animals were behaviorally scored for signs of the chronic phase weekly, were tested on the rotarod weekly, and underwent activity monitoring on days 85, 116 and 264 pi. Animals were bled via the saphenous vein of the leg on days 73, 110, 177, and 266 pi for antibody (Ab) to virus and Ab to myelin protein analyses. Mice

were euthanized at Day 277 pi with pentobarbital and perfused with PBS followed by 10% formalin.

Statistical Analysis

Analyses of variance (ANOVAs) were conducted on the data. Where possible, baseline measures were used as a covariate, and analyses of covariance (ANCOVAs) were conducted instead. Bonferroni t-tests, Duncan's multiple range tests, and means comparisons were used for post hoc analyses. For non-normally distributed data, a Mann-Whitney U test was used. Correlation matrices (Pearson's bivariate) were computed on select dependent measures to calculate the inter-relationships between the dependent variables. A p value of 0.05 or less was considered significant in all cases.

Results

Acute Phase

Body Weights. As depicted in Figure 11A, there was a main effect of sex prior to restraint stress, such that males weighed more than females, $F(1,19) = 23.02$, $p = 0.0001$. No other baseline differences existed. During the restraint stress period, there were significant main effects of sex and restraint stress on body weights, as well as significant sex X stress, sex X day pi, stress X day pi, and sex X stress X day pi interactions, all Fs ≥ 2.08, all ps ≤ 0.05. Males gained weight more quickly than females, and restraint stress mice gained less weight than nonstressed controls. Planned comparisons found that restraint stress decreased body weight to a greater degree in males than females. Following the

cessation of restraint stress (day 29 pi) there were significant main effects of sex and stress, such that males weighed more than females, and previously restraint stressed mice weighed less than nonstressed controls, both $\underline{F}s \geq 5.45$, both $\underline{p}s \leq 0.05$. These main effects were however qualified by sex X stress interaction, $\underline{F}(1,19) = 4.74$, $\underline{p} < 0.05$. Planned comparisons confirmed that in male mice previously restraint stressed mice weighed less than nonstressed controls, while in female mice there was no stress difference. By day 43 pi, there was no longer a main effect stress or a sex X stress interaction, both $\underline{F}s \geq 0.928$, both $\underline{p}s \leq 0.05$. The only significant difference was that of sex, such that male mice continued to weigh more than female mice, $\underline{F}(1,19) = 54.72$, $\underline{p} = 0.0001$.

Clinical Scores. As seen in Figure 11B, during the restraint stress period, there were significant main effects of sex and stress, and a sex X stress interaction on behavioral signs of encephaliytic-like symptoms in the acute phase, all $\underline{F}s \geq 6.897$, all $\underline{p}s \leq 0.05$. Overall, males had higher scores than females, and restraint stressed mice had higher scores than nonstressed controls, but both of these effects were qualified by the interaction between sex and stress. Planned comparisons found that restraint stressed males had higher scores than females, but this sex difference did not exist in nonstressed controls. Following the cessation of restraint stress, and prior to the onset of the chronic phase of disease (days 29-32pi), there were significant main effects of sex, stress, and day

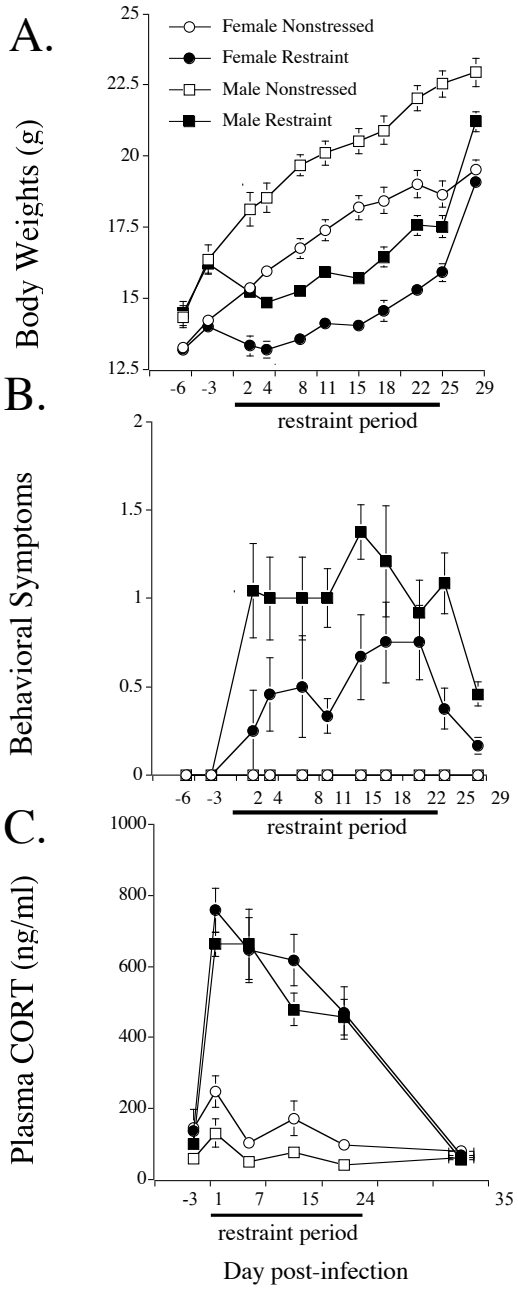

Figure 11. CBA Body Weights, Behavioral Signs of Illness, and Plasma CORT Levels in the Acute Phase. As shown in panel A, RST stress decreased body weights to a greater degree in male and female mice. By day 43pi, there were no differences in body weights between the RST and nonrestrained (NonRST) groups. RST stress increased behavioral symptoms to a greater degree in males that females (panel B). As shown in panel C, RST stress increased plasma CORT levels to a greater degree in female than male mice during the restraint period (days 1-24pi). Following the cessation of restraint, there were no differences between the RST and NonRST groups. All data are expressed as the mean $\pm$ SEM.

pi on behavioral scores, all Fs $\geq$ 5.429, all ps $\leq$ 0.05. Males had higher scores than females, previously restraint stressed mice had higher scores than nonstressed control mice, and scores decreased over time. There was also a significant sex X stress interaction and stress X day pi interaction, both Fs $\geq$ 5.429, both ps $\leq$ 0.05. When previously restraint stressed: males have higher scores than females and scores decreased over time. In nonstressed controls there was no sex difference, and scores did not change over time. By day 36pi, all mice displayed no behavioral signs of encephalytic-symptoms.

Plasma CORT Levels. There was a marginal baseline CORT level difference, such that female mice had higher CORT levels than male mice three days prior to infection, $F(1, 20) = 3.442$, $p = 0.078$. No other baseline differences were found. As depicted in Figure 11C, during the RST stress period, there were main effects of sex, stress, and day pi, but no significant interactions between the variables. CORT levels decreased over time, $F(3,60) = 7.81$, $p = 0.0002$. RST stressed mice had higher CORT levels than infected nonrestrained mice, $F(1,20) = 198.59$, $p = 0.0001$. Females continued to have higher CORT levels than males throughout the RST stress period, $F(1,20) = 4.19$, $p = 0.05$. Following the cessation of RST stress (Day 35 pi), there was no effect of sex or stress condition, nor a sex by stress interaction, all Fs $\leq$ 0.50, ps $\geq$ 0.49.

Food Intake. During the week prior to infection and restraint stress (day -6pi to day -3pi), there was a significant main effect of sex, $F(1,4) = 8.74$, $p < 0.05$, such that males consumed more food than females (Figure 12A). No other baseline differences were detected. During the first few nights of restraint stress

(day -3pi to day 2pi, and day 2pi to day 4pi), there were main effects of stress and sex, both Fs > 11.57, both ps < 0.05. Independent of condition, males consumed more food than females. Restraint stress reduced food intake across sex, as compared to nonstressed controls. During the last week of restraint stress (day 22pi to 25 pi) and following the cessation of the four week restraint stress period (day 25pi to day 29pi), there were also main effects of stress, both Fs ≥ 15.05, both ps ≤ 0.05. At this point, however, restraint stressed animals consumed more food than their nonstressed controls. No other differences were found.

Sucrose Preference. The only difference in sucrose preference existed at day 23 pi (Figure 12B), where restraint stressed mice had a greater sucrose preference than nonstressed controls, $F(1,4) = 19.57$, $p < 0.05$. At all other time points no significant differences between groups were found. A repeated measures design also found no significant effect of day pi. Given that sucrose preference is assessed per cage of mice, the small sample size (2 cages per condition) may have contributed to a lack of power indetecting significant effects on this measure. However, the significant finding on day 23 pi coincides with the increased sucrose preference seen in restraint stressed SJL mice, as compared to nonstressed controls.

Spontaneous Activity. See Figures 13A and 13B for horizontal and vertical activity, respectively. There were significant main effects of sex and stress, and a sex by stress interaction, on horizontal activity, all Fs ≥ 4.074, ps < 0.05. Females had greater horizontal activity than males. Stressed animals had decreased

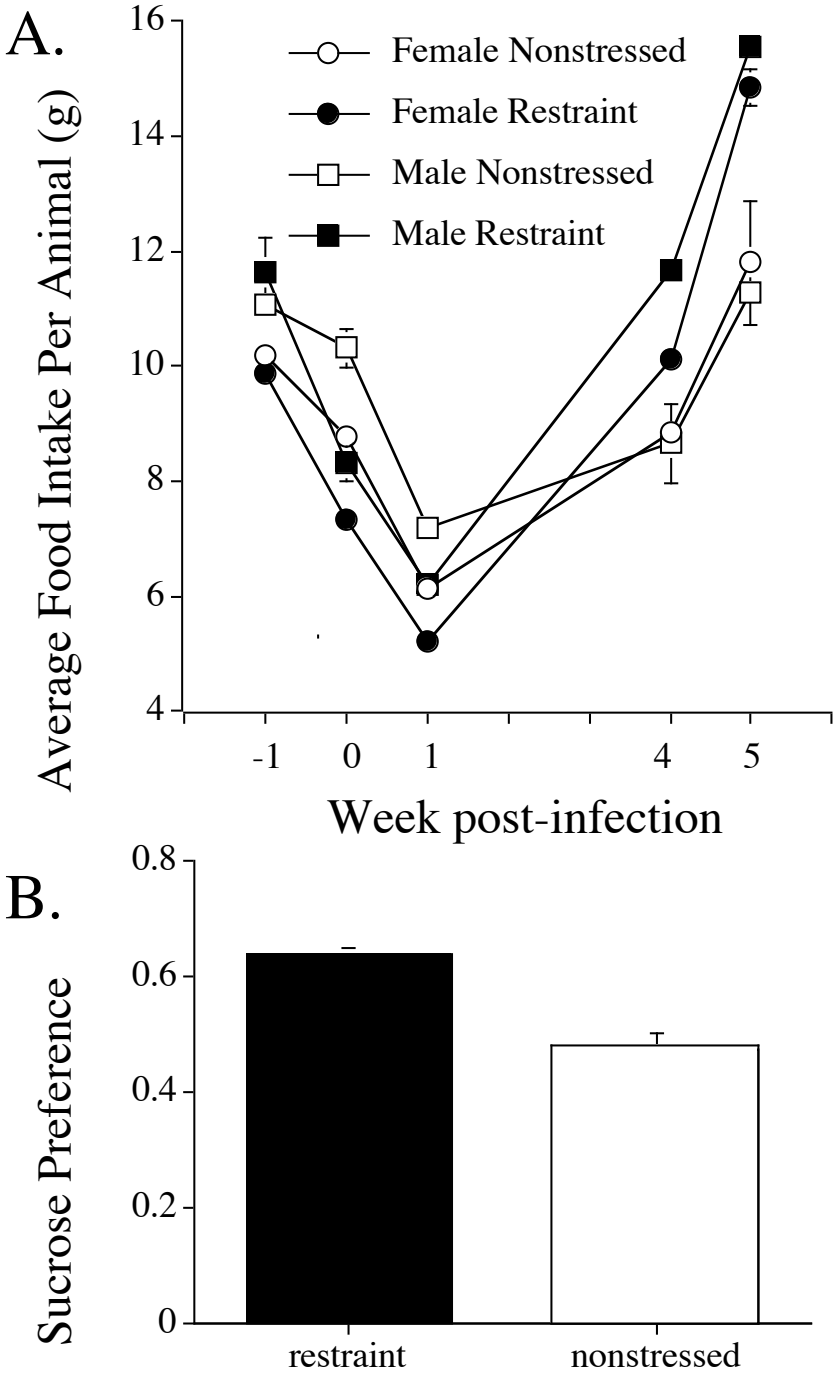

Figure 12. CBA Food Intake and Sucrose Preference in the Acute Phase. RST stress decreased food intake day-3-2pi and 2-4pi, while it increased food intake day 22-25pi and 25-29pi (panel A). RST stress increased sucrose preference on day 23pi (panel B).

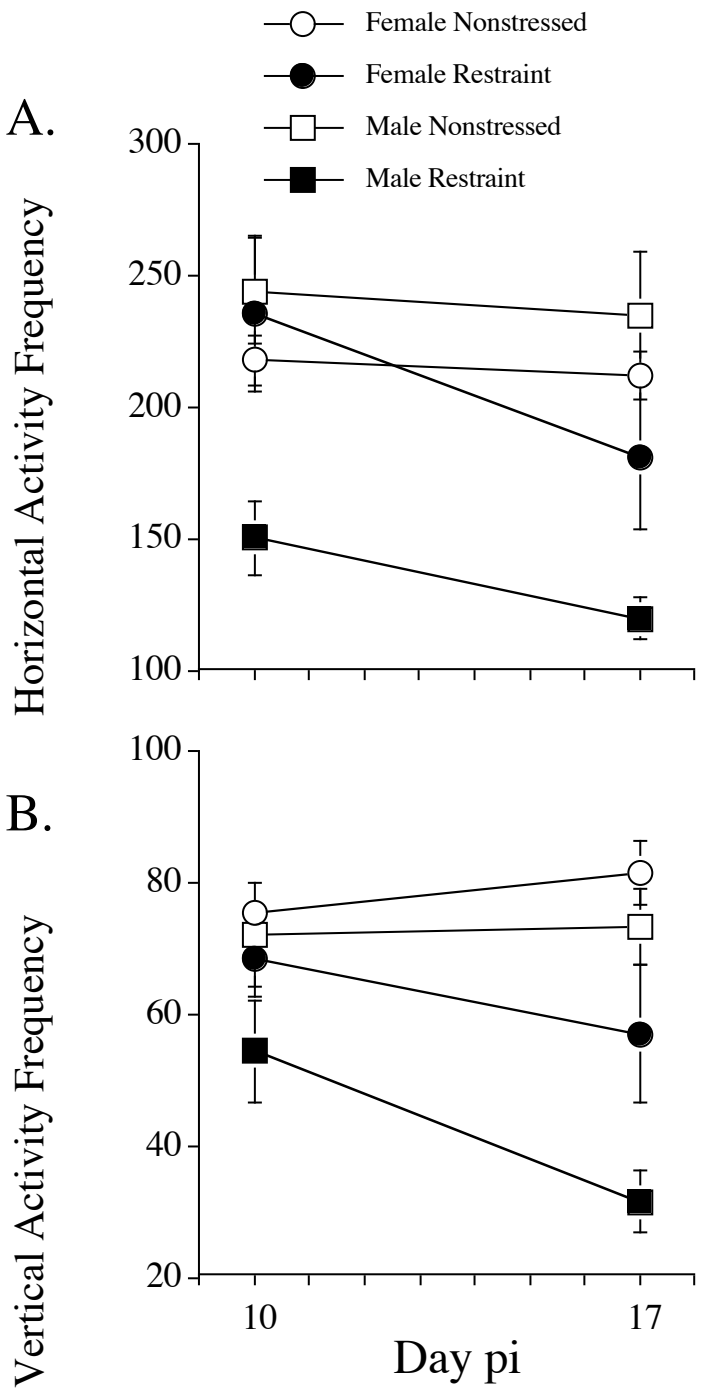

Figure 13. CBA Horizontal and Vertical Activity in the Acute Phase. RST stress decreased horizontal activity in males only (panel A) and vertical activity (panel B) in male and female CBA mice. All data are expressed as the mean $\pm$ SEM.

activity as compared to nonrestrained mice. Both of these main effects however, were qualified by the interaction between sex and stress, such that restraint stress decreased horizontal activity in males only. On the vertical activity measure (the sum of leaning, rearing and jumping frequencies), there were significant main effects of sex, stress, and a stress by day pi interaction, all $\underline{F}s \geq 4.612$, all $\underline{p}s < 0.05$. Females had greater vertical activity than males. RST stressed animals had decreased vertical activity as compared to nonrestrained mice. From day 10 pi to day 17pi, vertical activity decreased, but in only the restraint stressed animals.

Summary. The present study replicates the pattern of results previously observed in male CBA mice (Campbell et al., 2001; Satterlee et al., 2001; Faulkner et al., 2003; Welsh et al., 2004) in both males and females. However, unlike our prior study (Campbell et al., 2001), we did not see as severe mortality or behavioral signs of illness. In the current study, none of the mice died. Due to the high rate of mortality seen in Campbell and colleagues (2001), the degree of confinement in our restraint tubes was changed to be less severe. In the previous study, restraint tubes were 2-3 cm in diameter, 8 cm in length, and opaque. In the present study, transparent 60 ml syringes were used, with a diameter of 3 cm and a length of 10 cm. This was done to increase the survival rate of animals throughout the 4 weeks of restraint stress, and thus increase the possibility that the mice would be able to enter the chronic phase of disease. Even with this change, RST stress still had a significant effect on both male and female CBA mice during the acute phase of Theiler's virus infection, decreasing

body weights, food intake and activity levels (an indication of increased illness; Barak, 2002), while increasing behavioral signs of illness, plasma CORT levels, and sucrose preference (consistent with previous findings that stress increased the preference for sweet food; Badiani et al., 1996; Ely et al., 1997).

Chronic Phase

Behavioral Data. Previous RST stress had sex dependent effects on body weights, behavioral signs of the chronic phase of disease, and rotarod performance, such that previously RST males had heavier body weights, better rotarod performance and lesser behavioral signs of the chronic phase as compared to nonstressed control males, while the opposite pattern was observed in females. However, overall vertical and horizontal activity levels did not appear to be sensitive to the sex-dependent stress effects. The shorter duration of our session (10 min versus the 72 h used by McGavern, et al., 1999) may have decreased our ability to detect a significant effect of disease on this measure.

As depicted in Figure 14A, there were significant main effects of sex and day pi on body weights, both $\underline{F}s \geq 28.686$, both $\underline{p}s \leq 0.0001$. Males consistently weighed more than females and all mice gained weight over time. However, these main effects were qualified by a significant three-way sex X stress X day pi interaction, $\underline{F}(20, 340) = 2.224$, $\underline{p} = 0.002$. Whereas previously restraint stressed

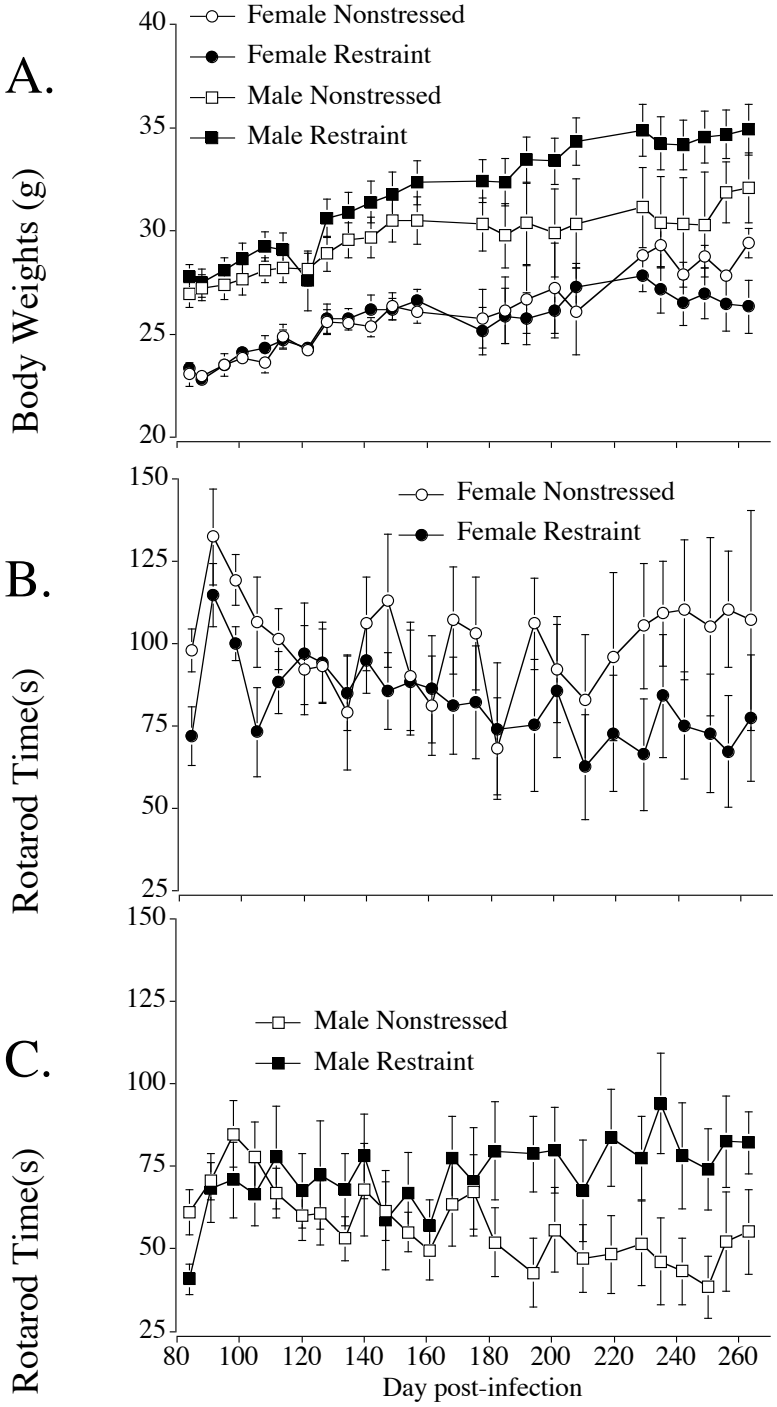

Figure 14. CBA Body Weights and Rotarod Time in the Chronic Phase. Previous RST increased body weights in male mice while it decreased body weights in female mice (panel A). Previously RST male mice had better rotarod performance at days 194, 235, and 250 pi than nonstressed male mice (panel C), while there was no significant difference in the female mice (panel B). All data are expressed as the mean $\pm$ SEM.

females gained less weight over time as compared to nonstressed control females, previously restraint stressed males gained more weight over time as compared to nonstressed control males.

As shown in figure 14B and 14C, there was a main effect of day pi on rotarod performance that was qualified by a three-way sex X stress X day pi interaction, both $\underline{F}s \geq 1.714$, both $\underline{p}s \leq 0.05$. T-tests confirmed that previouslyrestraint stressed male mice had better rotarod performance at days 194, 235, and 250 pi, all $ps \leq 0.05$. No other differences were found. Thus, previous restraint stress improved rotarod performance in the males, but had no effect on the females.

For behavioral signs of the chronic phase (Figure 15A), there was a significant main effect of day pi, $\underline{F}(12, 204) = 28.274$, $\underline{p} = 0.0001$, and a marginally significant sex X stress interaction, $\underline{F}(1,17) = 4.172$, $\underline{p} = 0.0569$. Independent of stress condition and sex, mice showed an increase in behavioral signs of the chronic phase of the disease. Planned comparisons found that this marginal difference was due restraint stressed females tending to have higher scores than males, but when nonstressed no sex differences were observed. The primary dimension in our chronic phase scoring system, wobbly gait, was analyzed separately (Figure 15B). There was a significant effect of day pi and a sex X stress interaction on wobbly gait, both $\underline{F}s \geq 4.54$, both $\underline{p}s < 0.05$. Over time, wobbly gait increased for all groups. In males, restraint stress decreased wobbly gait as compared to nonstressed controls, while in females there was a slight trend for restraint stress to increase wobbly gait.

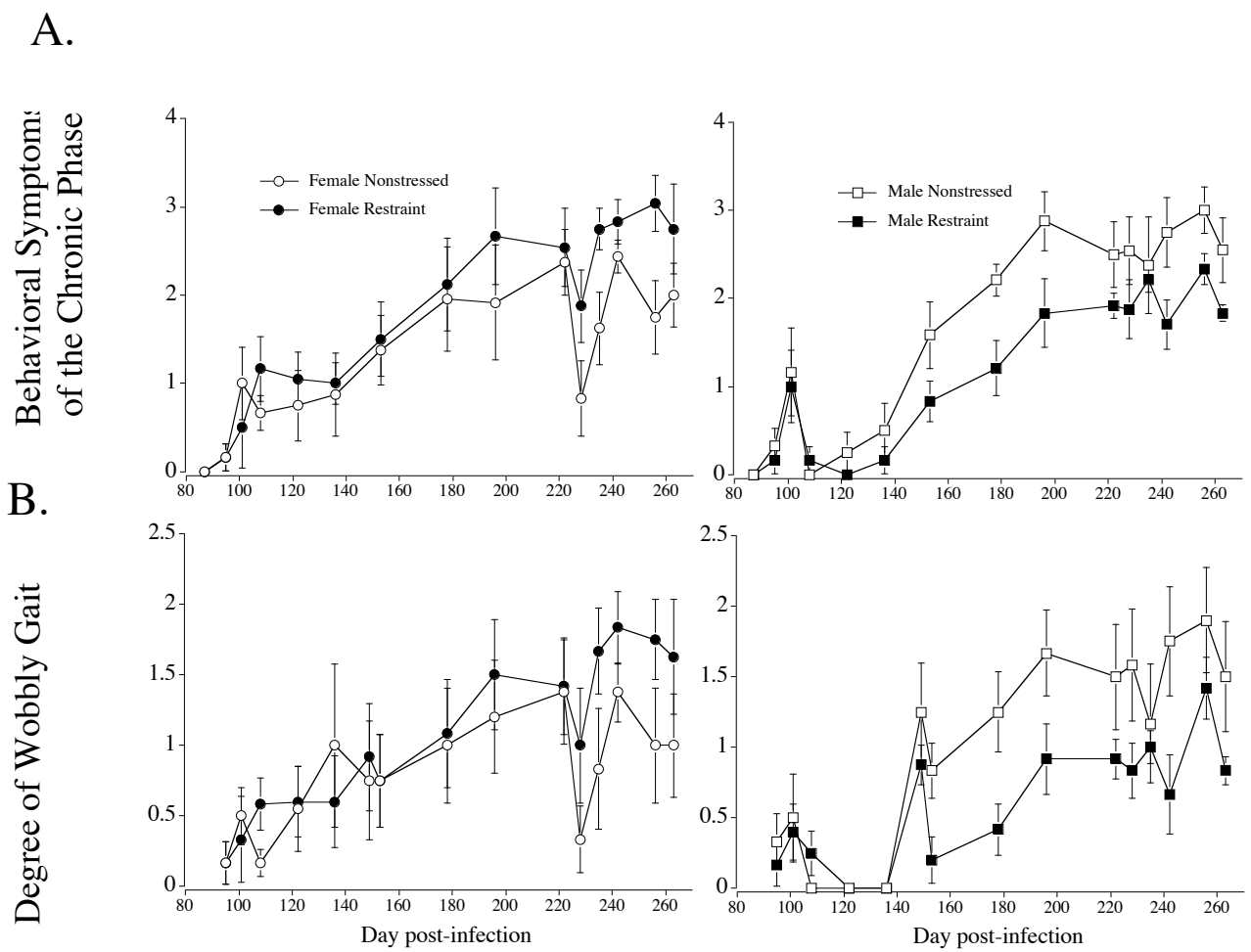

Figure 15. CBA Behavioral Symptoms and Wobbly Gait in the Chronic Phase. There was a tendency for female RST mice to have higher scores than male RST mice. In males, RST decreased wobbly gait as compared to nonstressed controls, while in females there was a slight trend for RST to increase wobbly gait. All data are expressed as the mean $\pm$ SEM.

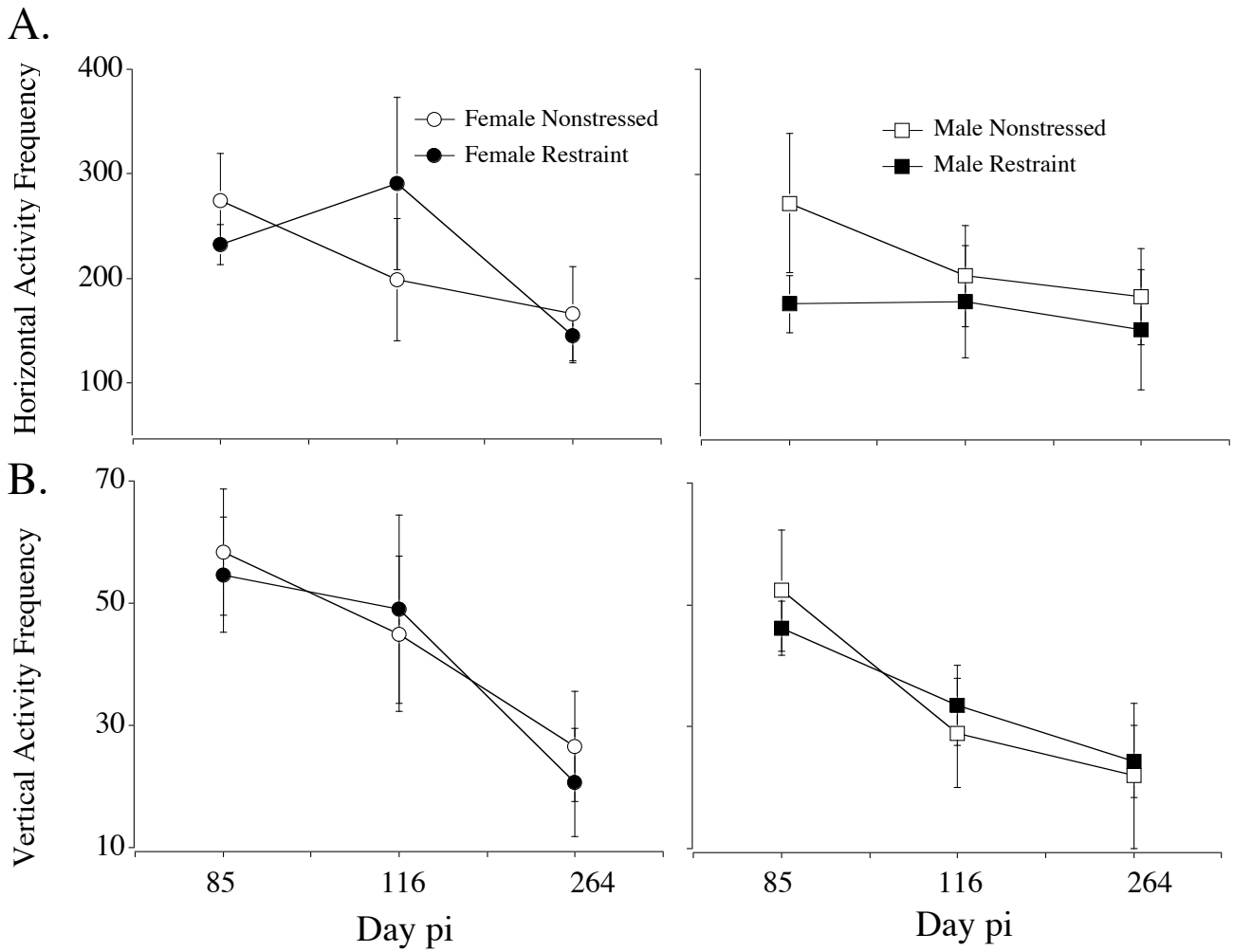

Figure 16. CBA Horizontal and Vertical Activity in the Chronic Phase. Horizontal activity decreased from day 85 pi to day 116 pi in nonstressed mice but did not decrease until day 264 pi in previously restraint stressed mice (panel A). There was no effect of stress or sex on vertical activity (panel B). However, consistent with previous reports on TVID, vertical activity decreased in all groups as the disease progressed. All data are expressed as the mean $\pm$ SEM.

There was a main effect of day pi, $F(2, 26) = 3.706$, $p < 0.05$, and a marginal interaction between stress and day pi, $F(2, 26) = 3.058$, $p = 0.064$, on horozontal activity. See Figure 16A and 16B for horizontal and vertical activity levels across days post-infection. All groups decreased horizontal activity over time. Planned comparisons found horizontal activity decreased from day 85 pi to day 116 pi in nonstressed mice but did not decrease until day 264 pi in previously restraint stressed mice. Restraint stress seemed to have delayed the decrease in horizontal activity over time. There were no effects of stress on vertical activity. There was, however, a main effect day pi, $F(2, 30) = 19.727$, $p < 0.0001$ Across all groups, vertical activity decreased as the disease progressed.

Taken together, the behavioral data collected during the chronic phase for CBA mice (body weights, behavioral signs of the chronic phase, wobbly gait, rotarod performance, and spontaneous activity) suggests that RST stress during the acute phase not only exacerbated the acute phase of the disease, but also the influenced the development of the chronic demyelinating condition. While restraint stress generally seemed to exacerbate the disease process in females, it was found to lessen indications of the chronic phase of disease in males. Restraint stressed males displayed increased body weights, rotarod performance, and horizontal activity levels, while decreased behavioral symptoms of the chronic phase of disease and wobbly gait

Histological Analysis of Lesions. Analyses of the inflammatory lesions within the spinal cord confirmed what was suggested by the behavioral data: in

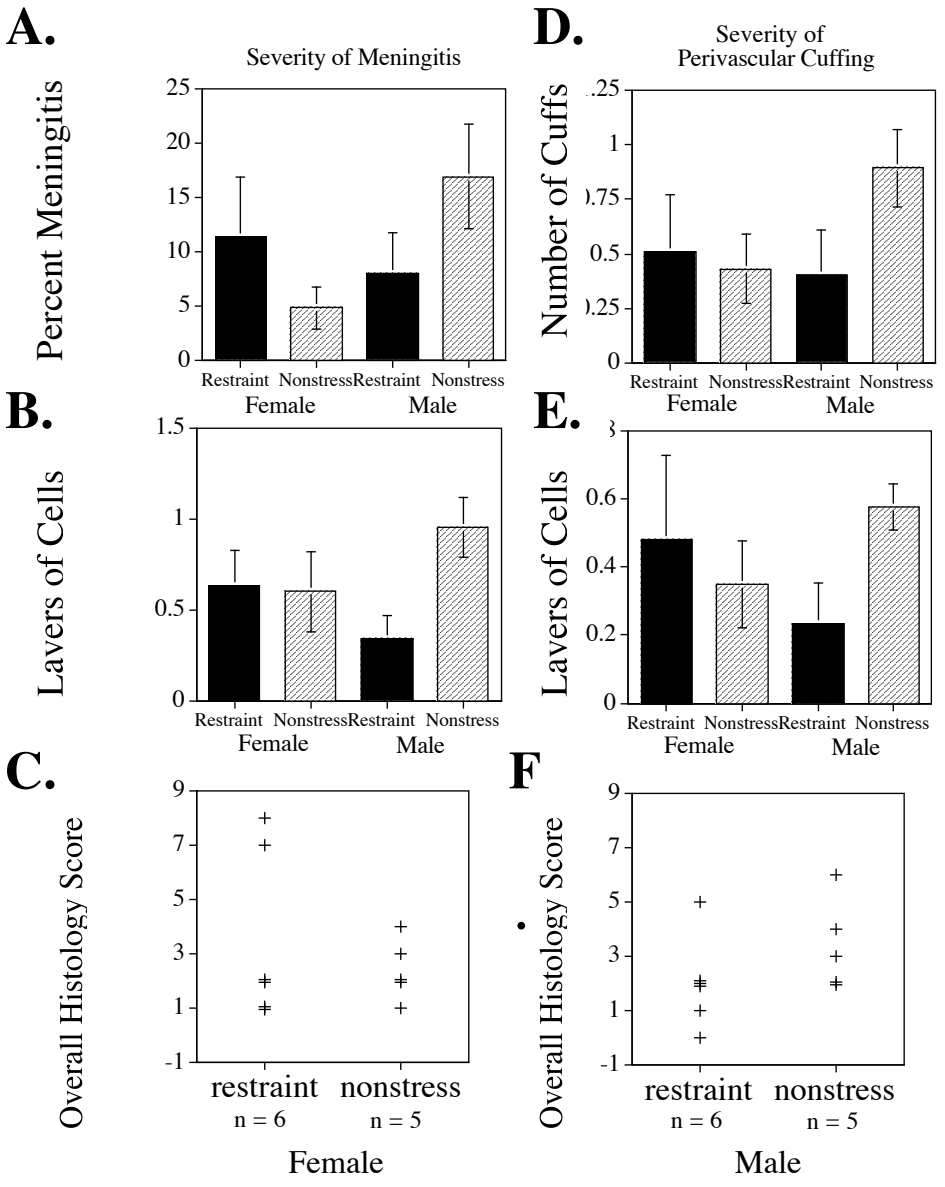

Figure 17. CBA Inflammatory Histological Lesions. While there was a trend for RST males to have decreased numbers of perivascular cuffs(D), numbers of cells in the cuffs(E), and percentage meningitis (A) as compared to nonstressed males, RST males had significantly fewer layers of cells in the meninges (B) than nonstressed males. Data are expressed as the mean $\pm$ SEM. A Mann-Whitney U test found the overall inflammation in RST males to be less than nonstressed males (F). Overall inflammation (C&F) was computed by assigning a score to each variable based on the quarter that the value was in (lowest 25% = 1 to highest 25% = 4). The two perivascular cuffing variable scores were then summed, as well as the two meningitis variable scores. The numbers represent two animals that had the same score.

the chronic phase of the disease male previously restraint stressed mice displayed less symptoms than their nonstressed controls (Figure 17A-F). Although there was a trend towards a sex by stress interaction for the percentage meningitis (Figure 17A), F (1,14) = 3.281, p = 0.09, the number of perivascular cuffs (Figure17D), F (1,14) = 1.791, p = 0.20, and the number of layers in the cuffs (Figure17E), F (1,14) = 2.830, p = 0.11, no main effects of stress or sex or interactions between stress and sex were significant for these measures, all Fs $\leq$ 3.28, all ps > 0.05. Post-hoc analyses found that difference between the restraint stressed and nonstressed males was responsible for the trends. However, withregard to the layers of cells in the meninges, there was a significant stress by sex interaction, F (1,14) = 5.538, p < 0.05. Post-hoc analyses confirmed that male restraint stressed mice had fewer cell layers in the meninges than nonstressed male mice, p < 0.05. No other group differences were significant. Due to the fact that a small portion of the CBA mice developed severe symptoms of the chronic phase of the disease (36%), there was low power to detect an effect with an analysis of variance. Given this, and the non-normal distribution of the data, the nonparametric statistic, a Mann-Whitney U test, was performed to detect any significant differences between the male stressed and nonstressed groups, and between the female stressed and nonstressed groups. The Mann-Whitney U test found a significant difference between the male stressed and nonstressed groups, such that previously restraint stressed mice had less histological lesions than nonstressed males. However, no significant

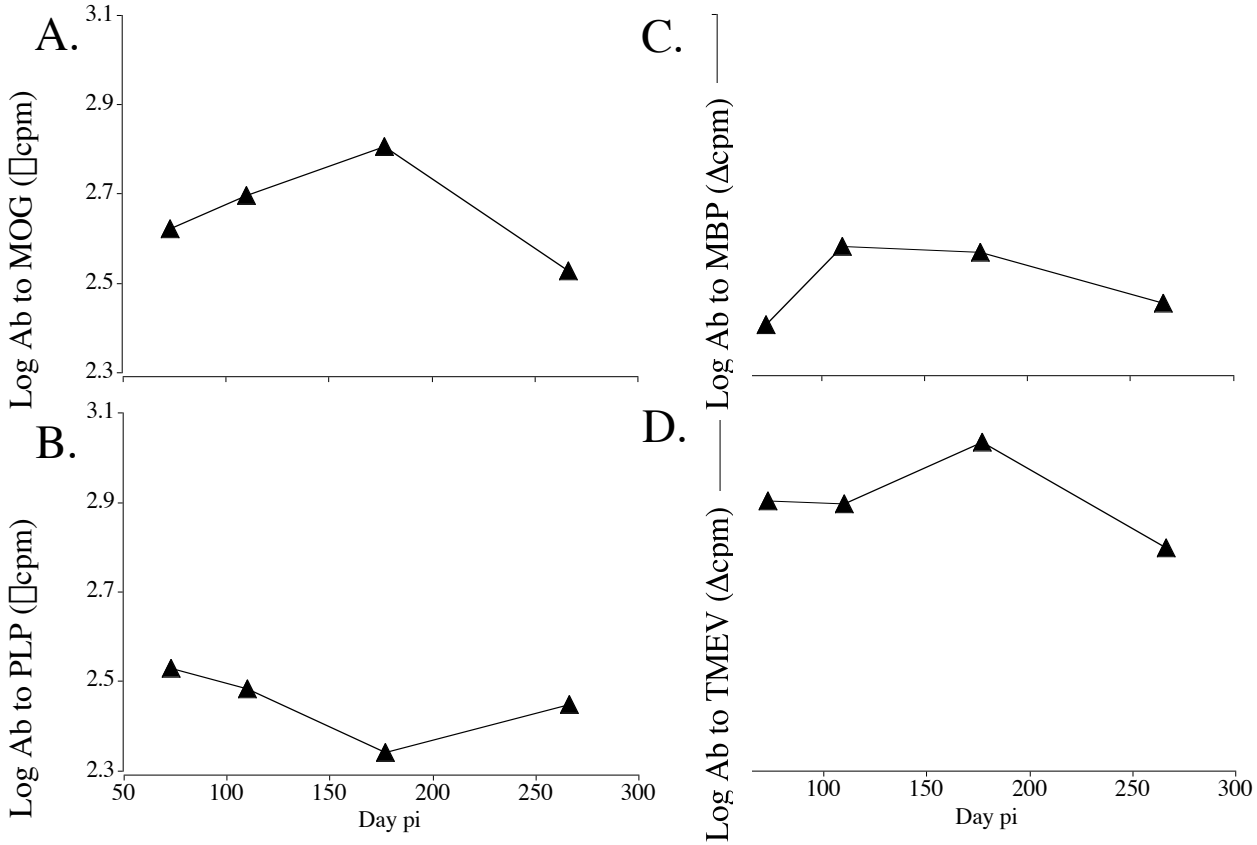

Figure 18. CBA Ab Levels to MOG, MBP, PLP< and TMEV. Ab to MOG33-55 levels increased from day 110 to day 177 pi, and then dropped at day 266 pi to levels below that seen at day 110pi (panel A). Ab to PLP139-151 levels were lower at day 177pi than all other time points (panel B). Ab levels to MBP were greater on days 110 and 177 pi than day 73 pi (panel C). Ab levels to TMEV were higher at day 177 pi than both days 73 and 110 pi., and levels dropped at day 266 pi to levels below that of day 73pi (panel D). The average cpm across dilutions is presented. No sex or stress differences were found.

difference was detected in the females. This pattern of data for the males and females is presented in Figures 17C and 17F.

Plasma Antibody Analyses. Plasma Ab levels to myelin components (MOG33-55, PLP139-151, and MBP) and to Theiler's virus were measured at days73, 110, 177, and 266 pi. As depicted in Figure 18A, a significant main effect of day pi was found for Ab to MOG33-55, $F(3,27) = 7.037$, $p = 0.001$. Post-hoc analyses found that ab to MOG33-55 levels increased from day 110 to day 177 pi, and then dropped at day 266 pi to levels below that seen at day 110pi. Previous RST stress did not impact this variable, and no other differences were found, all ps > 0.05. A significant main effect of day pi was also found for Ab to PLP139-151 levels, $F(3,36) = 5.80$, $p = 0.002$ (Figure 18B). No other effects were significant Post-hoc analyses found that the ab to PLP139-151 levels were lower at day 177pi than all other time points. As seen in Figure 18C, a significant main effect of day pi was found on Ab to MBP, $F(3,15) = 4.64$ $p < 0.05$. Post-hoc analyses found that ab levels to MBP were greater on days 110 and 177 pi than day 73 pi, all ps < 0.05. There was also a marginal decrease in MBP ab levels from day 177 pi to day 266 pi, $p = 0.77$. As seen with the ab to myelin components, a significant main effect of day pi was found on Ab to Theiler's virus, $F(3,33) = 12.44$, $p = 0.0001$ (Figure 18D). Post-hoc analyses found that ab levels to Theiler's virus were higher at day 177 pi than both days 73 and 110 pi. In addition, ab levels to Theiler's virus dropped at day 266 pi to levels below that of day 73pi. No other significant differences were found for Ab to Theiler's virus levels, all ps > 0.05. Thus, antibody levels do not reflect the stress effects

on disease progression, suggesting that changes in antibody production do not mediate the effects of restraint. Nevertheless, these findings are important because they characterize the autoantibody response and observed autoantibodies to PLP139-151, MOG33-55 and MBP in the late demyelinating phase of the disease.

Inter-relationships Between Dependent Variables

A correlation matrix was computed on acute phase dependent variables. Baseline CORT was significantly negatively correlated with baseline body weight, such that as weight increased, CORT level decreased. These baseline measures were not, however, significantly correlated with measures taken during restraint stress (body weights, CORT levels, behavioral symptoms, activity levels). The dependent measures taken during the restraint stress period were instead correlated with each other. Table 7 presents the correlations between summary variables: high behavioral score, initial weight loss, high CORT level, average horizontal and vertical activity at day 10 and 17pi. The correlation values for the summary variables high behavioral score, initial

Table 7. Correlation matrix for acute phase variables.

Dependent Measure	Day pi	Base-line Wt.	Base-line CORT	High Behav. Score	Initial Wt. Loss	High CORT
Day pi		**-3**	**-3**	**NA**	**NA**	**NA**
Baseline Weight	**-3**	1.0				
Baseline CORT	**-3**	**-.54****	1.0			
High Beh. Score	**NA**	.08	.07	1.0		
Initial Wt. Loss	**NA**	.01	.02	**.79****	1.0	
High CORT	**NA**	-.09	.09	**.87****	**.73****	1.0
Horizontal Activity	**10**	-.09	-.69	**-.47***	**-.46***	-.39^
	17	-.01	-.21	**-.61***	-.38^	**-.50***
Vertical Activity	**10**	-.25	.24	**-.48***	**-.46***	**-.47***
	17	-.22	.17	**-.63****	**-.61****	**-.59****

* = significant at p≤ 0.05 ** = significant at p≤ 0.01 ^= marginally significant at p≤ 0.10

There were very rarely significant correlations between acute and chronic phase variables (data not shown). The sex dependent effects of restraint stress on the chronic but not the acute phase likely account for this. When separate correlation matrices were run for male and female mice, again no significant correlations were found. The small sample size of 12 male and 12 female mice for this analysis did not provide enough power to detect any significant relationships. In contrast, a correlation matrix computed to determine the inter-weight loss, and high CORT are of similar magnitude to all of the behavioral scores, body weights and CORT levels throughout the restraint stress period.

Table 8. Correlation matrix for histology and chronic phase variables. There were significant correlations between histology variables and rotarod after day 105pi and behavioral symptoms after day 153pi.

DV	Day pi	Avg. Number PVCs	Avg. Layers in PVC	Avg. Percent Meningitis	Avg. Layers in Meninges
Rotarod Time	105	-.48*	-.43*	-.55**	-.43*
	112	-.51*	-.49*	-.43*	-.52*
	120	.46*	-.45*	-.36	-.41^
	126	-.56**	-.53*	-.47*	-.50*
	134	-.44*	-.37	-.40	-.32
	140	-.65**	-.51*	-.57**	-.46*
	147	-.31	-.31	-.23	-.32
	154	-.50*	-.44*	-.44*	-.42^
	161	-.58**	-.53*	-.44*	-.43*
	168	-.60**	-.65**	-.49*	-.65**
	175	-.35	-.41^	-.29	-.40^
	182	-.60**	-.67**	-.39	-.70**
	194	-.69**	-.67**	-.60**	-.62**
	201	-.70**	-.72**	-.52*	-.75**
	210	-.42*	-.46*	-.34	-.53*
	219	-.56**	-.60**	-.42^	-.62**
	229	-.68**	-.76**	-.53*	-.75**
	235	-.75**	-.80**	-.63**	-.78**
	242	-.70**	-.74**	-.61**	-.81**
	250	-.65**	-.70**	-.57**	-.70**
	256	-.77**	-.80**	-.64**	-.86**
	263	-.63**	-.66**	-.53*	-.68**
Chronic Behavioral Score	153	.21	.34	.27	.44*
	178	.31	.37^	.37^	.44*
	196	.39^	.45*	.43*	.51*
	222	.44*	.65**	.43*	.64**
	228	.36	.37	.51*	.42^
	235	.33	.46*	.31	.39^
	242	.18	.36	.27	.43*
	256	.53*	.61**	.57**	.67**
	263	.50*	.68**	.46*	.75**

* = significant at p≤ 0.05 ** = significant at p≤ 0.01 ^= marginally significant at p≤ 0.10

relationships between chronic phase variables found histological inflammatory lesion scores (number of perivascular cuffs, number of cell layers in the cuffs, percentage meningitis, number of cell layers in the meninges) to be highly correlated with the behavioral measures (behavioral symptoms of the chronic phase and rotarod time) collected in the mid to late chronic phase of disease (Table 8). Rotarod time significantly correlated with all four histology measures

Table 9. Correlation matrix for behavioral symptoms of the chronic phase with rotarod time and body weights.

DV	Day pi	Behavioral Symptoms of the Chronic Phase Scores								
Day pi		153	178	196	222	228	235	242	256	263
Rotarod Time	154	-.19	-.25	-.40	-.33	-.27	-.09	-.05	-.04	-.04
	161	.00	-.19	-.18	**-.42***	-.28	-.41^	-.07	-.30	-.20
	168	-.22	**-.41***	**-.42***	**-.56****	-.40^	**-.55****	-.24	**-.52***	**-.46***
	175	-.15	-.22	-.34	**-.45***	-.35	-.18	-.06	-.09	-.20
	182	-.08	-.31	-.21	**-.61****	-.15	-.35	-.15	-.30	-.40^
	194	-.32	**-.41***	**-.52***	**-.56****	-.39^	**-.47***	-.27	**-.45***	-.36
	201	**-.40***	-.39^	**-.54***	**-.67***	-.38^	**-.53***	-.31	-.42^	**-.48***
	210	-.29	-.25	**-.49***	**-.55****	-.26	-.30	-.21	-.19	-.26
	219	-.12	-.22	-.23	**-.55****	-.33	-.35	-.29	-.25	-.37^
	229	-.23	-.29	**-.52***	**-.67****	-.38^	**-.65****	-.35	**-.57****	**-.55****
	235	-.27	-.29	**-.45***	**-.61****	-.41^	**-.56****	-.35	**-.52***	**-.53***
	242	-.23	-.30	**-.48***	**-.63****	-.37^	**-.56****	-.30	**-.55***	**-.51***
	250	-.28	-.23	**-.48***	**-.61****	**-.52***	-.41^	-.32	-.38^	-.42^
	256	-.13	-.27	-.42^	**-.53***	-.37	**-.51***	-.30	**-.66****	**-.65****
	263	-16	-.13	-.41	**-.53***	**-.45***	-.31	-.30	**-.43***	-.41
Body Weights	157	**-.49***	-.29	-.32	-.28	-.06	-.15	**-.56****	-.12	-.20
	178	**-.72****	**-.57****	**-.58****	**-.54****	-.31	-.37^	**-.73****	-.35	**-.43***
	185	**-.80****	**-.60****	**-.65****	**-.63****	-.41^	**-.47***	**-.82****	-.42^	**-.51***
	192	**-.74****	**-.62****	**-.65****	**-.60****	-.38^	**-.51***	**-.81****	-.37^	**-.43***
	201	**-.81****	**-.60****	**-.72****	**-.64****	-.42^	**-.56****	**-.84****	-.41^	**-.48***
	208	**-.76****	**-.59****	**-.65****	**-.63****	-.37^	**-.49***	**-.82****	-.41^	**-.48***
	229	**-.64****	-.36^	**-.50***	**-.53***	-.35	**-.43***	**-.81****	-.35	-.41^
	235	**-.73****	**-.43***	**-.57****	**-.66****	**-.47***	**-.57****	**-.88****	**-.52***	**-.55****
	242	**-.68****	-.40^	**-.53***	**-.61****	-.36	**-.50***	**-.83****	-.41^	**-.50***
	249	**-.74****	**-.43***	**-.55****	**-.67****	**-.43***	**-.59****	**-.88****	**-.46***	**-.55****
	256	**-.61****	-.35	**-.44***	**-.57****	-.24	-.36	**-.80****	**-.43***	**-.53***
	263	**-.65****	-.40^	**-.50***	**-.60****	-.30	-.42^	**-.85****	**-.54***	**-.58****

* = significant at p≤ 0.05 ** = significant at p≤ 0.01 ^= marginally significant at p≤ 0.10

starting at day 105pi and throughout the chronic phase of disease. Behavioral symptoms of the chronic phase significantly correlated with histology beginning at day 153pi and throughout the chronic phase of disease. This effect was somewhat less consistent than the correlations with rotarod time. Body weights, ab levels to MOG, MBP, PLP and TMEV, and activity levels, however, did not have significant correlations with any of the histology measures.Additionally, there were significant correlations between behavioral symptoms of the chronic phase of disease and rotarod time and body weights (Table 9). Higher

behavioral symptoms of the chronic phase were associated with lower rotarod times and lower body weights. Body weights and rotarod time rarely correlated with each other, and all three of these variables very rarely correlated with ab levels or chronic phase activity levels (data not shown).

Discussion

Restraint stress applied during the first four weeks on infection with Theiler's virus exacerbated the acute CNS viral infection, decreasing body weights, food intake and activity levels, while increasing behavioral encephalitis-like symptoms and plasma corticosterone levels. This replicates the findings of Campbell and colleagues (2001) using male CBA mice and adds two additional measures: plasma corticosterone levels and spontaneous activity. The findings are summarized in Table 10. However, unlike our prior study (Campbell et al., 2001), we did not see as severe mortality or behavioral signs of illness. In the current study, none of the mice died. Reducing the severity of the confinement of the restraint tubes increased the survival rate of animals throughout the 4 weeks of restraint stress, and thus increased the possibility that the mice would be able to enter the chronic phase of disease. The current study also investigated sex differences, and found that exposure to restraint stress had more intense effects on the male mice than the female mice. During restraint, body weights for male mice dropped to a greater degree than female mice, and

Table 10. The effect of restraint on disease progression in CBA mice. Bold text (**increase** or **decrease**) indicate a significant effect; light text (increase or decrease) indicate and trend, and a dashed line (-----) indicates no effect of restraint stress.

Dependent Measure	Time Period	Male Mice	Female Mice	Sex Difference
ACUTE PHASE				
Body Weights	Baseline	NA	NA	M>F
	During Restraint	**DECREASE**	**decrease**	M>F
	D29pi	**decrease**	-----	M>F
	D43pi	-----	-----	M>F
Behav. Score	During Restraint	**INCREASE**	**increase**	MR>FR
	D29-32pi	**increase**	**increase**	MR>FR
	D36pi	-----	-----	-----
Plasma CORT	Baseline	NA	NA	F>M
	During Restraint	**increase**	**increase**	F>M
	D35pi	-----	-----	-----
Food Intake	Baseline	NA	NA	M>F
	D-3-2pi	**decrease**	**decrease**	M>F
	D2-4pi	**decrease**	**decrease**	M>F
	D22-25pi	**increase**	**increase**	-----
	D25-29pi	**increase**	**increase**	-----
Sucrose Preference	D23pi	**increase**	**increase**	-----
Horizontal Activity	During Restraint	**decrease**	-----	FR>MR
Vertical Activity	During Restraint	**decrease**	**decrease**	F>M
CHRONIC PHASE				
Body Weights	Chronic Phase	**increase**	**decrease**	M>F
Rotarod	Chronic Phase	**increase**	decrease	-----
Behav. Scores	Chronic Phase	decrease	increase	FR>MR
Wobbly Gait	Chronic Phase	**decrease**	increase	F>M
Horizontal Activity	D85,116,& 264pi	**increase**	**increase**	-----
Vertical Activity	D85,116,& 264pi	-----	-----	-----
# PVC	D277pi	decrease	-----	-----
Layers in PVCs	D277pi	decrease	-----	-----
% Meningitis	D277pi	decrease	-----	-----
Layers in Meninges	D277pi	**decrease**	-----	-----
Overall Histo. Score	D277pi	**decrease**	-----	-----
PLP, MBP, MOG, & TMEV Ab	D73, 110, 177, &266pi	-----	-----	-----

took longer to recover following restraint stress. Male restraint stressed mice had higher behavioral encephalitis-like symptom scores than females, and while females did not show a decrease in horizontal activity when restraint stressed, males did. The more intense behavioral effects of restraint stress seen in males, however, did not correspond to an increase in plasma corticosterone. In fact, female mice had higher CORT levels prior to restraint stress and infection, and continued to have higher CORT levels than males throughout the restraint stress period.

While restraint stress exacerbated the acute viral infection in both male and female mice, the pattern observed in the chronic phase was more complex and dependent on sex. Previously restraint stressed male mice developed less severe symptoms of the chronic demyelinating phase of disease as compared to nonstressed male mice: higher body weights, better rotarod performance, and more horizontal activity, while decreased behavioral symptoms of the chronic phase, wobbly gait and histological inflammatory lesions. When compared to nonstressed female mice, previously restraint stressed female mice showed a less robust pattern of results in the opposite direction: decreased body weights and increased behavioral symptoms of the chronic phase and wobbly gait. The opposing patterns of data for the male and female mice is presented in Table 10.

Though the pattern of results seen in the acute phase of the disease is very similar to that seen with SJL mice, there is quite a difference between the strains in the chronic phase of the disease. First of all while SJL mice began to show symptoms of the chronic phase of disease at 35 days pi, the CBA mice did

not show any clear indication of the chronic phase of disease until roughly 150 days pi. In addition to the later onset, the incidence of the chronic phase also varied. Coinciding with previous reports (Olesak et al., 2004; Simas & Fazakerley, 1996) only about one third of the CBA mice developed severe symptoms of the chronic phase of the disease, compared to 100% of the SJL mice. The impact of previous restraint stress on the chronic phase of disease likewise impacted the two strains differentially. Across both males and females, the disease course of SJL mice was exacerbated by previous restraint stress. This exacerbation was clear on behavioral signs of the chronic phase of disease, rotarod performance and histological inflammatory lesions of the spinal cord. When previous restraint stress did have a robust impact CBA mice, it did so in the opposite direction. Previously restraint stressed male CBA mice had lesser behavioral scores of the chronic phase and histological inflammatory lesions of the spinal cord, better rotarod performance, and increased horizontal activity and body weights. The impact of stress on the female CBA mice was more similar to that in SJL mice, but to a lesser degree. While sex differences were not observed in the SJL mice, they were apparent in the CBA mice.

Differences in the impact of stress on Theiler's virus infection between strains may be due to variability in either their responsivity to stress or their immunological background. The amount of restraint stress for SJL mice was reduced to eight hours per night because a pilot study (as well as previous unpublished findings from our lab) determined that uninfected SJL mice could not tolerate the twelve hours of nightly restraint stress used in the CBA mice.

Even with this reduction in the duration of the nightly restraint stress sessions, plasma CORT levels in the SJL mice were significantly higher than that of the CBA mice. An ANOVA confirmed that SJL mice had higher plasma CORT levels at baseline and during the restraint stress period, both $\underline{F}s > 7.2$, both $\underline{p}s < 0.05$. This difference in stress reactivity may differentially impact the immune response to Theiler's virus.

However, SJL and CBA mice have different immunological backgrounds without the experimental administration of a stressor. Welsh, Tonks, Borrow and Nash (1990) described the differences in immunological responses between SJL and CBA mice. The mechanism for demyelination varies across the two strains. SJL mice have a strong DTH response to virus, which results in the massive recruitment of macrophages into the CNS. Macrophages nonspecifically secrete substances such as proteases which cause bystander destruction of myelin and some degeneration of axon sheaths. In CBA mice, there is a lower DTH response to virus, less cellular infiltration, and the loss of myelin is not accompanied by axonal severance. Autoimmune processes may play a bigger role in CBA mice than in SJL mice, which show bystander destruction of myelin. In addition to the lesions being myelin or oligodendrocytes specific, ab to myelin components shows up earlier in the disease in CBA mice.

Susceptibility to Theiler's virus persistence and TVID has been linked to genetic differences between strains of mice. Monteyne, Bureau, and Brahic (1997) reviewed the genetic links to viral persistence of TMEV and susceptibility

to TVID. Lipton and colleagues (1977) found early on that when looking at inbred mouse strains, some were very susceptible to viral persistence and demyelination (SJL and DBA/2), some were intermediately susceptible (C3Hand AKR; CBA from Welsh et al., 1990), and some were able to clear the virus from the CNS, being resistant to the demyelinating phase of the disease (BALB/c and C57BL/6). While viral persistence and demyelination are under multigenomic control, one locus clearly linked to TVID susceptibility is the H-2 complex (Lipton et al., 1984). Using H-2 congenic mice on a C57BL/10 background, Rodriquez and colleagues (1985; 1990) concluded that resistance is a dominant trait and is found in mice with the haplotypes *b, d,* and *k.* Mice with the haplotypes *f, p, q, r, s,* and *v* are susceptible to viral persistence and demyelination. Bureau and colleagues (1992) looked at viral RNA levels in the spinal cord of various strains of mice and concluded that the haplotype *q* conferred a high level of susceptibility to viral persistence, while *b* conferred resistance. CBA mice have the H-2^k locus (Welsh et al., 1990) and SJL mice have the H-2^s locus (Monteyne et al., 1997). This H-2D region within the MHC influences susceptibility through it's effects on viral persistence (Bureau et al., 1992). The H-2 gene codes for a class I molecule, and suggests a key role for CD8+ CTL responses in clearing the infection (Bureau et al., 1992). CD8+ cells have been found necessary (Borrow et al., 1992) and sufficient (Nicholson et al., 1996) to clear Theiler's virus infection. Depleting mice of CD8+ cells with thymectomy and monoclonal antibodies prior to infection leads to increased viral titers in the CNS and increased demyelination in the chronic phase

(Borrow et al., 1992). Passive transfer of CD8+ cells from resistant strains of mice to susceptible strains confers resistance (Nicholson et al., 1996). Further evidence of the importance of CD8+ CTL activity comes from the finding that H-2D restricted CTL activity is present in the spleen of C57BL/6 at day 2 pi and increases greatly thereafter, while in SJL mice it does not appear until day 7 pi and remains at low levels for several months (Monteyne et al., 1997). It has also been suggested that TMEV specific CD8+ CTL activity might be a mechanism of killing oligodendrocytes in the chronic phase, thus leading to demyelination (Lindsley et al., 1991).

Other non-H-2 genes have been implicated in the susceptibility to viral persistence and demyelination in TVID (for reviews see: Monteyne et al., 1997; Oleszak et al., 2004). The T*crb* locus on chromosome 6, which codes for the T-cell receptor, has been linked to susceptibility to demyelination (Melvold et al., 1987). Welsh and colleagues (1990) suggested that in contrast to SJL mice, the T cell repertoire of CBA mice is more effective at the recognition of Theiler's virus because a portion of the animals are able to clear the virus from the CNS. Another gene implicated in viral persistence of TMEV is a region on chromosome 10 close to the I*fng* locus (Bureau, et al., 1993). IFN-γ is known for it's antiviral and immunomodulatory roles. Indeed, monoclonal antibodies directed against IFN-γ applied to resistant mice increased the persistence of TMEV in the white matter of the spinal cord, and the amount of demyelination (Rodriguez et al., 1995).

The H-2, T*crb*, and I*fng* associated genes all have their effect on susceptibility to demyelination at least partially through modulating virus persistence in the CNS. Nicholson and colleagues (1995) proposed that viral persistence may be necessary but not sufficient for susceptibility to the demyelinating disease in Theiler's infection; other genes mediate later steps which lead to inflammation and demyelination. Kappel, Melvold and Kim (1990) investigated the role of the H-2 locus in susceptibility to demyelination in crosses between SWR/J (H-2^q), C57L/J (H-2^b)and SJL (H-2^s). They found that sex influences susceptibility to demyelination in certain genotypes. Thus, sex and genetic determination of viral persistence can interact to determine susceptibility to demyelination.

In the current study, restraint interacted with sex to determine the susceptibility to the demyelinating disease in a single strain, CBA mice. Sex differences were not, however, observed in the impact of restraint stress on SJL mice (Chapter I). This sex difference may be due in part to either sexual dimorphism of the immune system or the stress systems. Females have a more robust humoral and cellular immune response to males. Additionally, sex hormones are able to modulate the immune response. While progesterone, androgens, and high levels of estrogen increase Th2 humoral immunity and decrease Th1 cellular immunity, low levels have the opposite effect. Females also have a more robust stress response, showing higher baseline CORT levels, and greater increases of CORT to ACTH and stress. This is possibly due to the reduced negative feedback of CORT on the HPA axis. Estrogen also stimulates

CRH synthesis and potentiates the actions of NE. Serotinergic regulation of ACTH and CORT may also differ between the sexes. To complicate the picture further, stress suppresses estrogen, progesterone and androgen levels. The complex nature of the interaction between sex and stress may help to explain the variety of effects found when investigating the impact of sex and stress on TVID. Alley and colleagues (2003) found male SJL mice to have more severe symptoms than females. Hill and colleagues (1998) found the opposite in SJL mice. The current study's findings (Chapter II) were similar to Alley in that SJL male mice had higher behavioral symptoms of the chronic phase, worse rotarod performance, and lower horizontal activity levels, but did not differ in inflammatory lesions of the spinal cord. Kappel and colleagues (1990), found that sex interacted with strain to determine susceptibility. The current study detected a sex difference in the susceptibility to the chronic phase only when restraint stressed (Chapter III). Restraint stressed females showed a slight exacerbation, while restraint stressed males displayed a reduction in symptomatology.

How could exacerbation of the acute disease lead to lesser symptomatology and inflammatory lesions in the chronic phase with male CBA mice? Separate mechanisms may be involved in clearing the early neuronal infection and the later viral persistence within the white matter. Thus, disrupting one may not necessarily disrupt the other. While NK cells and secretion of IFN-□/□ are essential for viral clearance from the grey matter during early infection, the activity of CTL cells and the secretion of IFN-□ may be

the most important players in limiting the viral spread and late persistent infection in the white matter (Monteyne et al., 1997). This may help to explain why even though restraint stress in the acute phase with male CBA mice has been found to decrease NK cell activity and increase viral titer in the acute neuronal infection stage of Theiler's infection (Campbell et al., 2001; Welsh et al., 2004), the current study has found that previous restraint stress during the acute phase with CBA male mice, in contrast, reduces behavioral symptomatology and histological lesions of the chronic demyelinating phase of infection.

Immunosuppression during the acute viral infection has likewise had contrasting effects on the development of the acute viral infection and chronic demyelinating phase of Theiler's infection (Lipton & Dal Canto, 1977). Administration of immunosuppressive treatments (cyclophosphamide and rabbit anti-mouse thymocyte serum) during the first 1-3 weeks of infection exacerbated the acute viral infection while it reduced susceptibility to the chronic demyelinating phase of TVID. During the acute viral infection, there was increased neuronal necrosis and microglial cell proliferation, longer maintenance of high viral titers, and a greater spread of the viral antigen into the neocortex and hippocampal complex. In contrast, the same immunosuppression treatment produced dramatically reduced mononuclear inflammatory cells in the meninges and spinal cord white matter, prevented demyelination, and resulted in no detection of virus antigen normally present in the meninges and white matter of the spinal cord. It is possible that the current findings in male CBA mice (Chapter III) and the findings by Lipton and Dal Canto (1977) both

illustrate the trade off between potential encephalitis by acute neuronal viral infection and the development of demyelinating disease by persistant viral replication in the macrophages, monocytes, and glia in the white matter. The immune response early on is necessary for viral clearance from the CNS, but there is also reason to believe that the immune system is not only *involved* in the chronic phase of disease, but may actually trigger the persistent viral replication and thus the chronic demyelinating phase of the disease. Following the acute neuronal infection, some of the primary sources of persistently infected cells are macrophages and monocytes which may travel to the CNS and deliver the virus to the white matter. Another possibility is that an inflammatory response in the CNS does not necessarily deliver the virus to that location, but possibly triggers the reactivation of replication. Blakemore and colleagues (1988) investigated the sequence of changes that resulted in demyelination in CBA mice. They concluded that inflammation in the CNS may be a prerequisite for the resurgence of viral replication in the chronic phase of the disease that initiates the demyelination process.

In conclusion, the current findings in male and female CBA mice provide more data for how the complex interaction between genetics, sex, exposure to a pathogen such as TMEV, and environmental factors such as stress determines the susceptibility to an autoimmune demyelinating disease such as MS. Stress is obviously having significantly differential effects on the diverse genetic and immunological backgrounds. Unlocking the mystery of this complex intersection of variables lies in determining how stress impacts the various

immune responses to TMEV in acute and chronic disease, across sex and strain of mice.

CHAPTER IV

HISTOLOGICAL ANALYSIS OF THE EFFECTS OF RESTRAINT STRESS ON MALE AND FEMALE SJL AND CBA MOUSE INFLAMMATION, DEMYELINATION, AND AXONAL LOSS FOLLOWING THEILER'S VIRUS INFECTION

Introduction

Restraint stress during the acute phase of Theiler's virus was found to worsen the disease course of the subsequent chronic demyelinating phase of disease in male and female SJL mice: worsened rotarod performance, higher behavioral symptoms of the chronic phase, and greater inflammatory lesions in the spinal cord, as determined by H&E staining. Male SJL mice were also found to have decreased rotarod performance and horizontal activity as compared to female SJL mice. The effect of restraint stress on CBA mice was dependent on sex. Restraint stress decreased the chronic demyelinating disease phase in males: increasing body weights, rotarod performance and horizontal activity, while decreasing the wobbly gait aspect of the behavioral symptoms of the chronic phase, and inflammatory lesions in the spinal cord (H&E). Restraint produced a less consistent pattern in the opposite direction in female CBA mice: decreasing body weights and horizontal activity. There was also a trend for restraint stress to decrease rotarod performance and increase wobbly gait in female CBA mice.

Multiple Sclerosis and Theiler's Virus induced Demyelination are characterized by inflammatory demyelinating lesions of the CNS (Oleszak et al., 2004). T cells, monocytes and macrophages, including resident microglia, are present in the perivascular space, meninges, and parenchema of the white matter of the CNS. Active MS and TVID lesions involve this inflammatory response in addition to demyelination. Loss of oligodendrocytes and axonal swelling and severance are also present in both, but there is a relative preservation of axons in demyelinated areas.

The purpose of the current study was to confirm that the stress-induced inflammatory spinal cord lesion changes seen in SJL (Chapter II) and CBA (Chapter III) mice were indeed associated with demyelination (as determined by Weil's myelin stain), and with relative preservation of axons (as determined by Holmes Silver stain). Direct comparisons between SJL and CBA inflammation, demyelination and axonal loss were also made, and the location of these lesion characteristics within the spinal cord was investigated.

Methods

Subjects

Male (n=12) and female (n=12) CBA mice were obtained from Harlan (Houston, TX) at three weeks of age. All mice were housed three per cage with food and water available ad libitum. Male and female mice were housed in separate rooms with separate ventilation systems. They were allowed to acclimate to their environment for one and a half weeks prior to infection,

during which time they were handled by all experimenters at least twice. All animals were housed in accordance with Texas A&M University and National Institutes of Health animal care guidelines.

Infection

The BeAn strain of Theiler's virus (obtained from Dr. H.L. Lipton, Department of Neurology, Northwestern University, Chicago, IL) was propagated and amplified in BHK-21 cells. The culture supernatant containing infectious virus was aliquoted and stored at -70^{0}C before use (Welsh et al., 1987). As in previous studies, mice were inoculated with 5x10^4 pfu of the BeAn strain of Theiler's virus intracranially into the right cerebral cortex (Welsh et al., 1987; Campbell et al., 2001) at 4.5 weeks of age.

Restraint Stress

Mice were restrained in their home cages, in 60 ml plastic syringes, drilled with holes for ample ventilation (Sheridan et al., 1991; Campbell et al., 2001). RST occurred for a duration of eight h (SJL) and twelve h (CBA), during the dark cycle, for 5 successive nights per week, with two days off in between weeks. The twelve h restraint duration was used in previous studies with CBA mice (Campbell et al., 2001), and the eight h duration in SJL mice was determined in a pilot study to be the maximum amount of restraint stress that uninfected SJL mice could tolerate. Both procedures produced significant behavioral and physiological changes in the acute and chronic phases on Theiler's virus infection (Chapters II and III).

Histological Analysis

Mice were euthanized with pentobarbital at 135 (SJL) and 277 (CBA) days pi, when both strains were clearly showing severe signs of the chronic demyelinating phase of disease. They were perfused via the left ventricle with PBS followed by 10% formalin in phosphate buffer pH 7.2, and processed as described in Campbell et al., (2001). Coronal spinal cord serial sections were stained with Hematoxylin and Eosin (H&E) for inflammation, Weil's Myelin stain for demyelination, and Silver stain for axonal loss. An experimenter blind to the subjects' conditions scored the H&E sections for the severity of inflammation (number of cell layers in the meninges or perivascular cuffs; microgliosis on a 5 point scale of increasing density of cells) and area of inflammation (percentage on meninges with inflammation, the number of perivascular cuffs, and the percentage of the white matter with microgliosis); the Weil's sections for severity of demyelination (0 = no myelin loss, 1 = moderate , 2 = severe loss of myelin) and area of demyelination (percentage of white matter without myelin); the Holmes Silver stain sections for severity of axonal loss (0 = no axonal loss, 1 = 25% of axons lost, 2 = 50% of axons lost, 3 = 75% of axons lost, 4 = no axons) and area of axonal loss (percentage of white matter with axon loss). The location of the lesions were also recorded (cervical, thoracic, lumbar, or sacral; dorsal, lateral, or ventral funiculi).

Procedure

A 2 (Strain) X 2 (Sex) X 2 (Stress) design was employed. Six subjects were placed in each group, counter-balanced by weight upon arrival, for a total of 48

subjects. All mice were infected. Half of all SJL and CBA mice were RST stressed one night prior to infection, and for the following 4 weeks. Previous studies from our laboratory (Campbell et al., 2001; Welsh et al., 2004) have found that restraint-induced changes in behavioral signs of illness, weight loss, NK cell activity, CNS viral titers, and histological CNS inflammation were selective to infected animals. Therefore, in the current study only infected animals were used to reduce animal numbers. Mice were euthanized at Day 135 (SJL) or 277 (CBA) pi with pentobarbital and perfused with PBS followed by 10% formalin.

Statistical Analysis

Analyses of variance (ANOVAs) were conducted on the data. Means comparisons were used for post hoc analyses. For non-normally distributed data, a Mann-Whitney U test was used. A p value of 0.05 or less was considered significant in all cases.

Results

Lesion Characteristics

In both SJL and CBA Theiler's virus infected mice, inflammatory demyelinating lesions within the spinal cord were present with relative axonal preservation. In the H&E stained spinal cord sections, inflammatory cells were visible in the meninges (meningitis), surrounding vessels within the white matter (perivascular cuffs), and within the parenchema of the white matter (microgliosis). The inflammatory lesions were primarily located within the

cervical and thoracic regions of the spinal cord. Too a lesser degree, inflammatory lesions were detected in the lumbar segments, and were rarely seen in the sacral segments. Inflammatory lesions were generally restricted to the ventral and lateral funiculi, but were occasionally observed in the dorsal funiculi, and could be either unilateral or bilateral in nature. Loss of myelin detected by the Weil's myelin stain was consistently overlapping with the areas of microgliosis. Additionally, the greater the density of cells in the microgliosis lesions, the more severe the loss of myelin. In some instances, demyelination was observed in locations close to meningitis and perivascular cuffing, but there was typically an infiltration of cells into the parenchema accompanying the demyelinated lesion. Only the severely demyelinated lesions had any significant reduction in axons, as detected by Holmes Silver stain. This reduction was never a complete loss of axons, thus there was always a relative preservation of axons. In less severely demyelinated lesions, there was often very minimal loss of axons. No axonal reduction was detected without the presence of demyelination. Likewise, no demyelination was detected without the presence of inflammation. The three characteristics of the lesions were almost always completely overlapping. Mild to moderate inflammation, however, was occasionally detected without demyelination or axonal loss.

SJL Histology

Figures 19 and 20 depict the amount and severity of inflammation, demyelination and axonal loss averaged across cervical, thoracic, and lumbar segments of the spinal cord in male and female restrained and nonrestrained

mice. ANOVAs were conducted on each lesion characteristic averaged across cervical, thoracic and lumbar segments. There were significant effects of stress and sex on the percent area meningitis (Figure 19A), both Fs $\geq$ 5.72, both ps < 0.05, such that stressed mice and males had higher levels. Stress also significantly increased the layers of cells in the meninges, F (1,16) = 9.55, p < 0.01. There was a marginally significant effect of sex, such that males had increased layers of cells in the meninges (Figure 19B), F (1,16) = 3.26, p = 0.089. The only effect of sex or stress perivascular cuffing (Figure 19C&D) was a marginally significant effect of stress increasing the number of cell layers in the cuffs, F (1,17) = 3.47, p = 0.080. There was a significant stress-induced increase in the percent area microgliosis (Figure19E), F (1,17) = 19.98, p < 0.01, and a marginally significant greater percent area microgliosis in males, F (1,17) = 3.27, p = 0.088, but no significant effects of stress or sex on the severity of microgliosis (Figure 19F), both Fs $\leq$ 2.30, both ps > 0.05. There were significant stress-induced increases in percent area demyelination (Figure 20A) and severity of demyelination (Figure 20B), both Fs $\geq$ 4.85, both ps < 0.05. Males had significantly higher percent area of demyelination, F (1,17) = 13.07, p < 0.01. The percent area of axonal loss (Figure 20C) was significantly increased by stress, F (1,17) = 10.55, p < 0.01. There was also a marginally significant stress-induced increase in the severity of axonal loss (Figure 20D), F (1,17) = 4.05, p = 0.060, however there were no effects of sex on either axonal loss measure, both Fs $\leq$ 2.26, both ps > 0.05. Table 11 summarizes the amount and severity of each

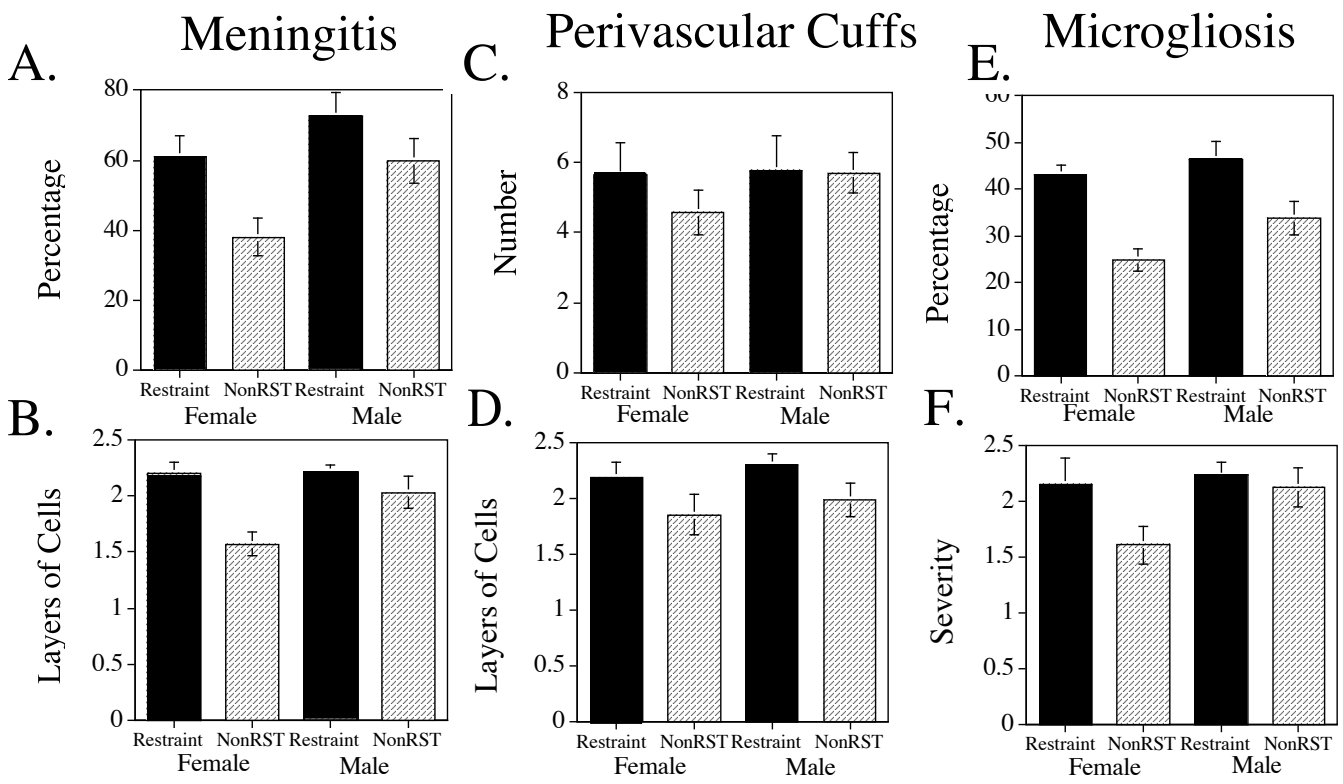

Figure 19. Average Inflammatory Lesions Across Cervical, Thoracic and Lumbar Spinal Segments in SJL Mice. Restraint stress significantly increased inflammatory lesions in SJL mice: percent area meningitis (A), layers of cells in the meninges (B), and percent area microgliosis (E), and marginally increased the layers of cells in cuffs (D). Males had significantly higher percent area meningitis (A), and marginally higher layers of cells in the meninges (B) and percent area microgliosis (E).

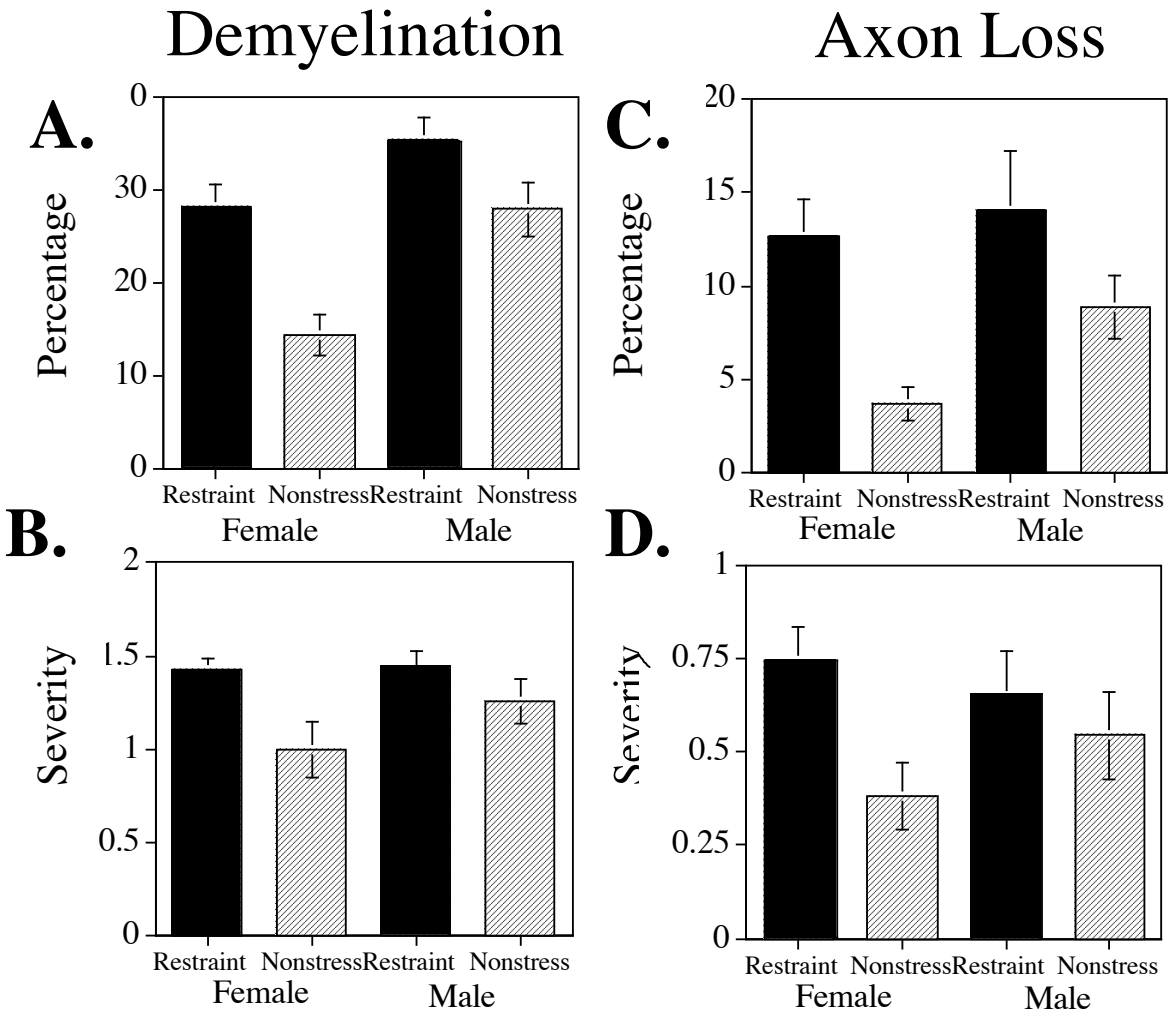

Figure 20. Average Demyelination and Axonal Loss Across Cervical, Thoracic and Lumbar Spinal Segments in SJL Mice. Restraint stressed SJL mice had significantly higher percent area of demyelination (A), severity of demyelination (B), percent area axonal loss (C), and a marginally significant higher severity of axonal loss (D). Males had significantly higher levels of percent area demyelination (A).

Table 11. The effect of restraint stress and sex on histological lesions in SJL mice. "**Increase"** or "**decrease"** indicate a significant effect of restraint stress and a horizontal line (-----) indicates no effect. M and F refer to male and female (combined restraint and nonstressed groups); MR and FR refer to male restraint and female restraint groups; MC and FC refer to male nonstressed and female nonstressed groups.

Histology measure	Spinal Location	Average Level MEAN (SEM)	Effect of Restraint Stress		Sex Differences
			Male	Female	
Average % Area Demyelination	Cervical	33.02 (2.91)	**increase**	**increase**	-----
	Thoracic	37.71 (3.18)	-----	**increase**	MC>FC
	Lumbar	6.25 (1.27)	-----	-----	M>F
	Sacral	0.33 (0.31)	-----	-----	-----
Average Severity Demyelination	Cervical	1.68 (0.11)	-----	-----	-----
	Thoracic	1.62 (0.08)	-----	**increase**	MC>FC
	Lumbar	0.50 (0.08)	-----	-----	-----
	Sacral	0.02 (0.02)	-----	-----	-----
Average % Area Axonal Loss	Cervical	10.83 (1.72)	**increase**	**increase**	-----
	Thoracic	16.45 (2.54)	-----	**increase**	MC>FC
	Lumbar	0.62 (0.29)	-----	-----	-----
	Sacral	0.00 (0.00)	-----	-----	-----
Average Severity Axonal Loss	Cervical	0.79 (0.11)	-----	-----	-----
	Thoracic	0.88 (0.12)	-----	**increase**	MC>FC
	Lumbar	0.09 (0.04)	-----	-----	-----
	Sacral	0.00 (0.00)	-----	-----	-----
Average % Area Microgliosis	Cervical	47.78 (3.71)	**increase**	**increase**	-----
	Thoracic	49.34 (3.16)	-----	**increase**	MC>FC
	Lumbar	11.45 (2.26)	**increase**	**increase**	M>F
	Sacral	2.03 (1.75)	-----	-----	-----
Average Severity Microgliosis	Cervical	2.36 (0.16)	-----	-----	-----
	Thoracic	2.69 (0.13)	-----	**increase**	MC>FC
	Lumbar	0.98 (0.14)	-----	-----	-----
	Sacral	0.10 (0.09)	-----	-----	-----
Average % Meningitis	Cervical	67.13 (4.44)	-----	-----	-----
	Thoracic	68.64 (4.57)	**increase**	**increase**	M>F
	Lumbar	30.56 (5.40)	-----	-----	M>F
	Sacral	2.65 (2.30)	-----	-----	-----
Average # Layers of Cells in Meninges	Cervical	2.31 (0.10)	-----	-----	-----
	Thoracic	2.37 (0.10)	-----	**increase**	MC>FC
	Lumbar	1.22 (0.14)	-----	-----	-----
	Sacral	0.06 (0.05)	-----	-----	-----
Average # of Perivascular Cuffs	Cervical	7.20 (0.44)	-----	-----	-----
	Thoracic	6.04 (0.43)	-----	-----	-----
	Lumbar	2.96 (0.49)	-----	-----	-----
	Sacral	0.00 (0.00)	-----	-----	-----
Average # of Layers of Cells in Perivascular Cuffs	Cervical	2.47 (0.14)	-----	-----	-----
	Thoracic	2.53 (0.19)	-----	-----	-----
	Lumbar	1.05 (0.15)	-----	-----	-----
	Sacral	0.00 (0.00)	-----	-----	-----

lesion characteristic separately for the cervical, thoracic, lumbar and sacral regions of the spinal cord, as well as the effect of restraint and sex differences on the lesions. The effects of stress and generally restricted to cervical and thoracic regions. Though both males and females show a stress-induced increase in spinal cord lesions, the effect was more consistent in females. Overall, males tended to have greater spinal cord lesions than females.

CBA Histology

Nine of the twenty-three CBA mice developed severe symptomatology of the chronic demyelinating phase of disease. One of the twenty-four CBA mice (female, nonstressed, infected) died without any indication of the chronic phase of disease (behavioral score = 0, normal body weight of 32.1g) before the histological analysis could be performed (day 196pi). Another female nonstressed mouse was sacrificed at day 196pi with severe signs of the chronic phase of the disease (high behavioral score of 5, low body weight of 15.4g) and histological analysis was performed. This mouse is one of the two female nonstressed mice that had inflammatory lesions of the spinal cord. Two male nonstressed mice died at day 249 and 263pi with severe signs of the chronic phase of the disease (high behavioral scores of 4.5 and 4, low body weights of 18.7 and 26.8g), and histological analyses were unable to be performed. Thus, of the twenty-one mice of which histological analysis was performed, only seven had inflammatory lesions of the spinal cord.

Separate ANOVAs were conducted on all of the histology measures for all of the segments of the spinal cord for CBA mice, however no significant

differences were found. Given the non-normal distribution of the data, a Mann-Whitney U test was performed. Mice were separated into categories based on the presence or absence of inflammatory demyelinating lesions in the spinal cord. Table 12 provides the number of mice in each experimental group that had lesions in the spinal cord. The Mann-Whittney U test confirmed that male nonstressed mice had greater demyelination, axonal loss, microgliosis, meningitis, and perivascular cuffing than male restraint stressed mice. No significant differences were found between restraint and nonstressed female groups. Thus, the effects of restraint stress on the inflammatory demyelinating lesions in CBA mice was sex dependent.

Table 12. Frequency of restraint and nonstressed CBA mice with histological lesions. Mice were categorized as having no significant lesions, or lesions in at least two segments of the spinal cord. Male nonstressed mice had significantly more of each lesion characteristic than restraint stressed males. No female differences were significant.

Histology Lesion Characteristic	Number of Mice With Lesions/Total Mice			
	Male		**Female**	
	Restraint	Nonstress	Restraint	Nonstress
Demyelination	1/6	2/4	2/6	2/5
Axonal Loss	0/6	2/4	2/6	2/5
Microgliosis	1/6	2/4	2/6	2/5
Meningitis	1/6	2/4	2/6	2/5
Perivascular	1/6	2/4	2/6	2/5

Unstressed SJL and CBA Histology Comparison

Further analyses were conducted on all of the nonstressed SJL and CBA mice that did develop inflammatory demyelinating lesions of the spinal cord

(SJL: 6 male and 6 female mice, CBA: 2 male and 2 female mice), to determine if the strain differences existed in not only the *incidence* of the chronic phase of disease (SJL: 100%, CBA: 55%), but also the severity or composition of the lesions. Figures 21 and 22 display the amount of demyelination, axonal loss, microgliosis, meningitis, and perivascular cuffing averaged across cervical, thoracic, and lumbar regions of the spinal cord for male and female nonstressed SJL and CBA mice. An ANOVA conducted on the percent meningitis (Figure 21A) found a significant effect of strain, $F(1,11) = 15.52$, $p < 0.01$, such that SJL mice had higher levels than CBA mice. There was also a marginally significant strain by sex interaction, $\underline{F}(1,11) = 4.24$, $\underline{p} = 0.064$. Means comparisons determined that in males, SJL mice had higher levels than CBA mice; in SJL mice, males had higher levels than females. Analyses on the number of cell layers in the meninges (Figure 21B) found a significant effect of sex and strain, both $\underline{F}s \geq 7.77$, both $ps \leq 0.05$. Males had a greater number of cell layers in the meninges than females, and SJL mice had a greater number than CBA mice. There was a significant strain difference in both the number of perivascular cuffs (Figure 21C) and the number of cell layers in the cuffs (Figure 21D), such that SJL mice had higher levels than CBA mice, both $\underline{F}s > 5.69$, both $\underline{p}s < 0.05$. A significant strain by sex interaction was found for the percent microgliosis (figure 21E), $F(1,12) = 5.24$, $p < 0.05$. Means comparisons determined that in males only, SJL mice had higher levels than CBA mice. No significant differences were detected for the severity of microgliosis (Figure 21F). An

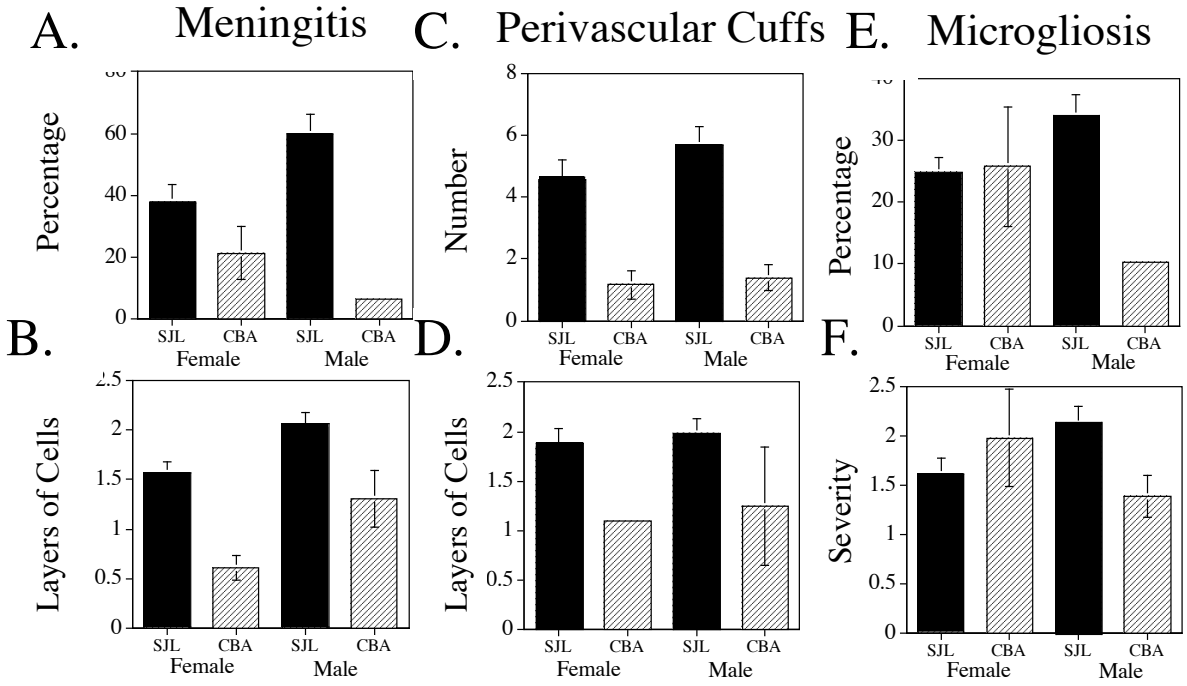

Figure 21. Average Inflammatory Lesions Across Cervical, Thoracic and Lumbar Spinal Segments in Nonstressed SJL and CBA Mice. SJL males had higher percent meningitis CBA males and in SJL mice, males had higher levels than females (A). Males had a greater number of cell layers in the meninges than females, and SJL mice had a greater number than CBA mice (B). SJL mice had greater numbers of perivacular cuffs (C), and more layers of cells in the cuffs (D) than CBA mice. SJL males had higher percent microgliosis (E) than CBA males. There were no differences on the severity microgliosis (F).

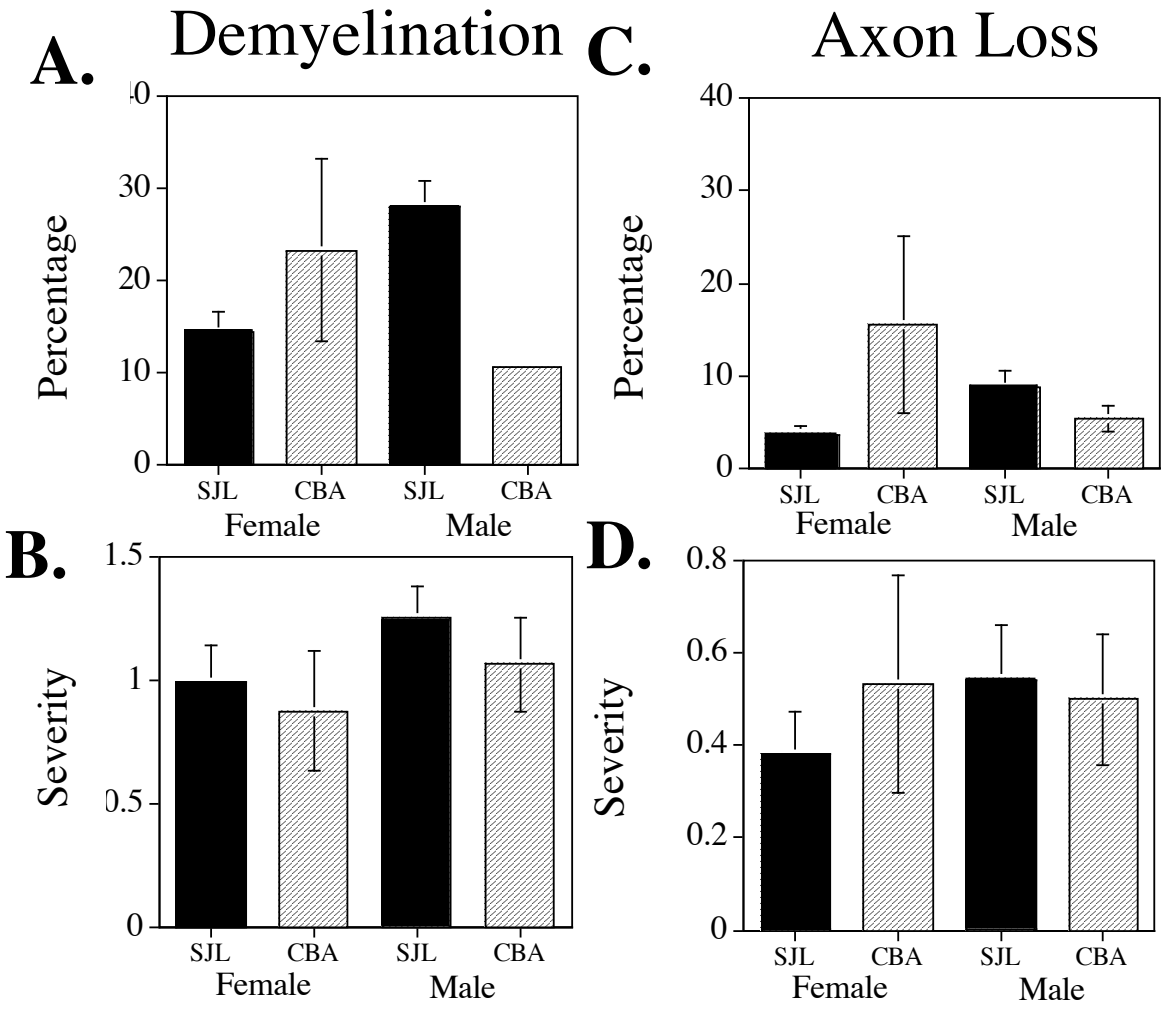

Figure 22. Average Demyelination and Axonal Loss Across Cervical, Thoracic and Lumbar Spinal Segments in SJL and CBA Nonstressed Mice. In males only, SJL mice had greater percent demyelination than CBA mice (A). In SJL mice only, males had greater percent demyelination than females. In females only, CBA mice had greater percent axonal loss than SJL mice (C). There were no differences in the severity of either demyelination (B) or axonal loss (D).

Table 13. Strain and sex differences in histological lesions of nonstressed mice. Bold letters, **SJL>CBA**, indicate a significant difference; parentheses, (SJL>CBA), indicate a marginal difference; a horizontal line, -----, indicates no effect.

Histology measure	Spinal Location	Strain Differences		Sex Differences	
		Male	Female	SJL	CBA
Average % Area Demyelination	Cervical	-----	-----	------	-----
	Thoracic	**SJL>CBA**	**CBA>SJL**	**M>F**	-----
	Lumbar	-----	-----	-----	-----
	Sacral	-----	-----		-----
Average Severity Demyelination	Cervical	-----	-----	-----	-----
	Thoracic	-----	**CBA>SJL**	**M>F**	-----
	Lumbar	(SJL>CBA)	(SJL>CBA)	-----	-----
	Sacral	-----	-----	-----	-----
Average % Area Axonal Loss	Cervical	-----	-----	-----	-----
	Thoracic	-----	**CBA>SJL**	**M>F**	-----
	Lumbar	-----	-----	-----	-----
	Sacral	-----	-----	-----	-----
Average Severity Axonal Loss	Cervical	-----	-----	-----	-----
	Thoracic	-----	**-----**	**M>F**	-----
	Lumbar	-----	-----	-----	-----
	Sacral	-----	-----	-----	-----
Average % Area Microgliosis	Cervical	-----	------	-----	-----
	Thoracic	**SJL>CBA**	-----	**M>F**	**F>M**
	Lumbar	**SJL>CBA**	**SJL>CBA**	-----	-----
	Sacral	-----	-----	-----	-----
Average Severity Microgliosis	Cervical	-----	-----	-----	-----
	Thoracic	**SJL>CBA**	-----	**M>F**	-----
	Lumbar	**SJL>CBA**	**SJL>CBA**	-----	-----
	Sacral	-----	-----	-----	-----
Average % Meningitis	Cervical	**SJL>CBA**	**SJL>CBA**	-----	-----
	Thoracic	**SJL>CBA**	-----	**M>F**	**-----**
	Lumbar	**SJL>CBA**	**SJL>CBA**	-----	-----
	Sacral	-----	-----	-----	-----
Average # Layers of Cells in Meninges	Cervical	-----	**SJL>CBA**	-----	**M>F**
	Thoracic	**SJL>CBA**	**SJL>CBA**	**M>F**	**-----**
	Lumbar	**SJL>CBA**	**SJL>CBA**	-----	-----
	Sacral	-----	-----	-----	-----
Average # of Perivascular Cuffs	Cervical	**SJL>CBA**	**SJL>CBA**	-----	-----
	Thoracic	**SJL>CBA**	**SJL>CBA**	-----	-----
	Lumbar	**SJL>CBA**	**SJL>CBA**	-----	-----
	Sacral	-----	-----	-----	-----
Average # of Layers of Cells in Perivascular Cuffs	Cervical	-----	-----	-----	-----
	Thoracic	**SJL>CBA**	-----	**M>F**	-----
	Lumbar	**SJL>CBA**	**SJL>CBA**	-----	-----
	Sacral	-----	-----	-----	-----

ANOVA detected a significant strain by sex interaction for the percent demyelination (Figure 22A), $\underline{F}\ (1,12) = 7.059$, $\underline{p} < 0.05$. Means comparisons

found that in males only, SJL mice had higher levels than CBA mice, and in SJL mice only, male mice had higher levels than females. No significant differences were found for the severity of demyelination (Figure 22B). Means comparisons used to follow up a marginal strain by sex interaction, $\underline{F}$ (1,12) = 4.124, $\underline{p}$ = 0.065, found that in females CBA mice had greater percent axonal loss (Figure 22C) than SJL mice, but there were no differences found for the severity of axonal loss (Figure 22D).

The statistically significant sex and strain differences for the separate cervical, thoracic, lumbar and sacral segments of the spinal cord are summarized in Table 13. There was an overall pattern for SJL males and females to have greater inflammation (microgliosis, meningitis, and perivascular cuffing) than CBA mice. This was most common in the thoracic and lumbar regions, but was also apparent in the cervical region. No sacral differences were detected on any measure. In the majority of animals, sacral lesions were not observed at all. The strain differences with demyelination and axonal loss did not always fit the pattern observed in the inflammatory measures that had SJL mice as having greater lesions than CBA mice. Much less consistently than with inflammation, male SJL mice had greater demyelination than male CBA mice, but no difference in axonal loss. In female mice, however, it was more likely for CBA mice to have greater demyelination and axonal loss than SJL mice. In SJL mice, males had higher levels of inflammation demyelination and axonal loss, but only in the thoracic region. Sex differences in CBA mice were less prevalent and less clear.

Discussion

The current study provides evidence that the inflammatory lesions within the spinal cord observed in SJL (Chapter II) and CBA (Chapter III) mice were indeed representative of inflammatory demyelinating lesions with relative preservation of axons. Consistently, there was an overlap of the areas of the spinal cord that had microgliosis, demyelination and axonal loss. Meningitis and perivascular cuffing that accompanied cellular infiltration into the parenchema, were also observed with the demyelinating lesions.

Strain differences were observed in the *incidence* of developing inflammatory demyelinating lesions and the *severity* of the inflammatory lesions. 100% of SJL mice developed inflammatory demyelinating lesions in the spinal cord by day 135pi. In contrast, only 30% of the CBA mice developed such lesions by day 266pi (44% nonstressed CBA, 25% restraint stressed). The level of the inflammatory measures (microgliosis, meningitis, and perivascular cuffing) were consistently higher in SJL mice as compared to CBA mice, especially in the thoracic and lumbar regions, and in male mice. Strain differences for demyelination were much less prevalent and less consistent: in males, SJL mice had higher levels than CBA mice, but in females the opposite was true. Strain differences in axonal loss were minimal, and when existed showed CBA mice as having greater axonal loss than SJL mice (females only). Thus, while SJL mice displayed greater inflammation, this did not necessarily result in greater demyelination and axonal loss.

Sex differences were consistently observed in SJL mice, such that males had higher levels of each lesion characteristic as compared to females, in the thoracic region of the spinal cord. This is similar to the findings from Alley andcolleagues (2003), but contradicts with others (Hill et al., 1998). Sex differences in CBA mice were minimal.

Strain and sex differences likewise occurred with the impact of restraint stress on inflammatory demyelinating lesions of the spinal cord. In SJL mice, previous restraint stress increased microgliosis, meningitis, perivascular cuffing, demyelination, and axonal loss in both males and females, but to a greater degree in females. In CBA mice, previously restraint stressed males actually had a lower incidence of having microgliosis, meningitis, perivascular cuffing, demyelination, and axonal loss than nonstressed males, while there was no significant effect of restraint stress on female CBA mice.

The chronic demyelinating phase of Theiler's virus manifests itself somewhat differently in SJL and CBA mice. CBA mice develop symptoms of the chronic phase of disease much later than SJL mice (Chapter II, III, and IV), with severe symptoms appearing at approximately day70pi for SJL mice, and day 190pi for CBA mice. Due to this substantial difference in the time post-infection that onset of the demyelinating disease occurs, direct comparisons between SJL and CBA mice may either reflect different stages in the disease process or strain differences in how the disease manifests itself. A comparison of SJL and CBA mice at the same day post-infection would not be comparing two groups of mice in the same stage of illness. At day 135pi, all of the SJL mice had severe

symptoms of the chronic phase of disease, while no CBA mice were showing symptomatology. A comparison of SJL and CBA mice when they are both displaying the same behavioral indications of the chronic phase of disease, as was done here, is a comparison done at substantially different times post-infection: day 135pi versus day 266pi. It is impossible to rule out time post-infection, or even age, as a mediator of the strain differences observed in inflammation. If the CBA mice were younger or if the virus had been in the spinal cord for 130 days less time, would the inflammation be similar to that of SJL mice? The mechanism of these strain and sex differences needs to be elucidated. However, data from this study make clear that genetics, sex, and the environment (e.g. stress) all play a role in the susceptibility to, and disease course of, Theiler's Virus Induced Demyelination.

CHAPTER V

SUMMARY AND CONCLUSIONS

Previously stressed SJL mice displayed an exacerbation of the chronic demyelinating disease, in both males and females. Stressed mice had increased behavioral signs of the chronic phase, decreased rotarod performance, and increased inflammatory demyelinating lesions in the spinal cord. Acute phase variables collected during the stress period, such as weight loss, behavioral encephalitis-like symptoms, and plasma corticosterone levels, were consistently correlated with disease progression in the chronic phase. This provides some of the first experimental evidence of a stress-induced exacerbation of an autoimmune demyelinating disease coinciding with reports from MS patients that stress precipitates the onset and relapses of MS.

Further research needs to be conducted to determine the mechanism of this stress-induced exacerbation of disease in SJL mice. Altering early immune responses to Theiler's virus with stress may have a cascading effect on later immune responses in the chronic demyelinating phase of disease. Cellular and humoral responses to virus and autoantigens in the chronic phase may be altered in response to the changed early immune responses. Alternatively, stress-induced suppression of the immune system during the early CNS viral infection may have reduced viral clearance from the CNS in the acute phase. This increased viral load in the acute phase may have translated into increased viral replication in the chronic phase. With more viral replication, a more

profound immune response would have been directed against viral and autoantigens.

With a different genetic background, the effect of stress were in the opposite direction of that seen in SJL mice. Previously stressed male CBA mice displayed an alleviation of symptoms in the chronic demyelinating phase of disease: increased body weights, increased rotarod performance, decreased behavioral signs of the chronic phase of disease, and decreased inflammatory demyelinating lesions of the spinal cord. Sex was also a determining factor in the stress effects in CBA mice. Previously restraint stressed female mice showed a less consistent pattern in the opposite direction: a mild exacerbation of the chronic demyelinating disease. Further research is needed to determine how stress-induced suppression of the immune system, leading to an exacerbation of the acute phase, could result in an alleviation of disease in the chronic demyelinating phase in male CBA mice. It is possible that the degree of the early CNS viral infection may not correlate with the severity of the chronic demyelinating phase. The mechanism of the sex-depenedent effects also needs to be investigated.

Stress during early Theiler's virus infection had strain and gender dependent effects on the development of the subsequent demyelinating phase of the disease. Susceptibility to developing MS and TVID appears to involve a complex intersection between genetic susceptibility, gender, exposure to a pathogen and stress.

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- Investigated the effects of stress on an animal model of Multiple Sclerosis, Theiler's Virus induced Demyelination in mice
- Behavioral assays of illness, emotional reactivity, and motor abilities; Mouse care and handling, anesthetic and drug administration, blood collection; Dissection, tissue collection, tissue preparation, sterile procedure; Radioimmunoassays, cell culture, immunohistochemistry, and histology of the central nervous system; Data collection, statistical analysis, graphical and written presentation

Behavioral Neuroscience Research Assistant, Texas A&M University, 1997-2000

- Investigated the underlying neurobiological structures for the impact of emotion on pain perception in rats
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Neurobiology Research Assistant, Southwestern University, 1996-1997

- Measured heat shock protein distribution in the crayfish nervous system following heat and surgery stress
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